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2-*p*-AMINOBENZENESULPHONAMIDO-4:6-DIMETHOXYPYRIMIDINE: EXPERIMENTAL EVALUATION

BY

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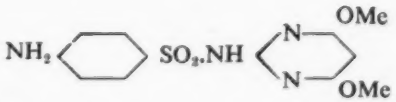
(Received January 16, 1947)

The introduction of an α-pyridyl residue into the molecule of sulphanilamide by Ewins and Phillips (1937) was a major advance in the development of sulphanilamide therapy, since not only was there produced a marked improvement in intrinsic antibacterial activity, but the new substance, sulphapyridine, was the starting point for an extensive research on the preparation of sulphanilamide drugs derived from other heterocyclic systems. The pyrimidine ring system has been particularly fruitful and at least three drugs derived from this nucleus are in common use: sulphadiazine, sulphamerazine, and sulphamezathine. They are characterized by antibacterial activity of a high order against a wide range of organisms, by rapid and efficient absorption from the gastrointestinal tract, and by a degree of persistence in the blood which provides economy in use. The work of Bell and Roblin (1942) suggests that the discovery of sulphanilamide drugs possessing a higher intrinsic antibacterial activity than that exhibited by the sulphapyrimidine group is becoming more remote. Any improvement in sulphanilamide therapy must therefore arise from research devoted to the production of drugs exhibiting reduced toxicity and enhanced persistence in the blood. We have interested ourselves for some time particularly in the latter aspect, and have found this property to be outstanding in sulphanilamides derived from 2-amino-4:6-dialkoxypyrimidines. A series of such compounds has been prepared (Rose and Tuey, 1946) and we propose to publish in due course detailed pharmacological and bacteriological findings on these substances as a class. This memoir is concerned with the parent compound 2-*p*-aminobenzenesulphonamido-4:6-dimethoxypyrimidine (sulphadimethoxypyrimidine), which is the most effective of the many homologues which have been examined.



While this paper was in preparation our attention was drawn to the researches of van Dyke, Tupikova, Chow, and Walker (1945), who, in the course of an

extensive study of sulphapyrimidines, have examined some of the 4:6-dialkoxy derivatives with which we have been concerned. In the main, these authors confirm our findings.

We describe the pharmacology and *in vitro* antibacterial properties of sulphadimethoxypyrimidine, the combination of these factors in therapeutic activity, the toxicity and the physico-chemical properties. A note on the unusual properties of the acetyl derivative of sulphadimethoxypyrimidine is included.

The clinical activity and pharmacology of the drug are now being investigated at Crumpsall Hospital, Manchester, and will be reported later by the workers concerned.

Pharmacology

The blood concentrations attained by sulphadimethoxypyrimidine have been examined in the mouse, rat, rabbit, dog, chick, sheep and calf. For data on the last two species we are indebted to Mr. J. Francis, of our Veterinary Research Laboratories.

In the mouse.—Absorption was examined by the standard technique already described (Rose and Spinks, 1946). Three mice received 250 mg./kg. by stomach tube as a 1 g./100 ml. solution of the sodium salt, and the drug was estimated at intervals in pooled tail blood by the method of Rose and Bevan (1944). In a series of 14 analyses of 0.05 ml. samples of blood containing 2 to 20 mg./100 ml., the mean recovery was 98.5 per cent ±standard deviation 6.95 per cent; limit of error for a probability level of 0.05, ±4.00 per cent.

The single experiment was repeated 22 times, so that 66 animals contributed to the mean results (Table I); in Fig. 1 the mean absorption curve is compared with that of sulphamerazine.

TABLE I

BLOOD CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE

250 mg./kg. in groups of 3 mice

Route of administration	Mean blood concentrations in mg./100 ml. after								
	20 min.	40 min.	1 hr.	1.5 hr.	2.5 hr.	3.5 hr.	5 hr.	7 hr.	24 hr.
Oral (22 expts.)	11.6	12.7	13.2	14.1	13.8	14.4	14.3	14.0	9.4
Intraperitoneal (13 expts.)	17.7	21.5	21.1	20.2	20.4	19.3	19.1	17.0	9.4
Subcutaneous (6 expts.)	14.1	19.1	18.8	19.9	17.0	20.6	20.3	18.6	7.7

In an earlier paper (Rose and Spinks, 1946) the expression C7, which is the time required for the blood concentration to fall from that attained 7 hours after dosing to two-thirds of this figure, was suggested as a convenient means of defining the rate of disappearance of a sulphonamide from the blood. Sulphadimethoxypyrimidine is clearly more persistent than sulphamerazine, the values

of C7 taken from the mean curves being 16.2 and 7.3 hours respectively. By obtaining the characteristic values from individual curves (Rose and Spinks, 1946) and submitting the results to statistical analysis, the difference is readily shown to be decisive ($P<0.01$). Sulphadimethoxypyrimidine is thus 2.2 times as persistent as sulphamerazine in mice, the term persistence being taken to mean the retention of free drug in the blood. The new drug reaches fairly high concentrations; the mean of the maximum concentrations from individual curves is 15.3 ± 1.25 mg./100 ml., which is higher than the corresponding figure for

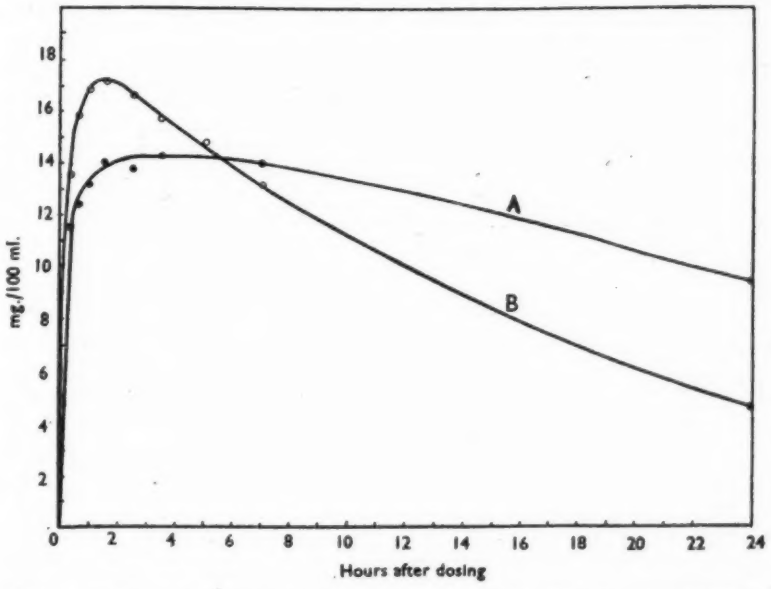


FIG. 1.—Blood concentrations in mice of sulphadimethoxypyrimidine (A) and sulphamerazine (B), following the administration of 250 mg./kg. orally.

sulphanilamide, sulphapyridine, or sulphathiazole, although lower than that for sulphamezathine, sulphamerazine, or sulphadiazine. Sulphadimethoxypyrimidine is rather slowly absorbed, the time at which the maximum blood concentration is attained, taken from the mean curve, being 220 minutes. Using the method of statistical analysis already described (Rose and Spinks, 1946), the drug can be shown to be significantly more slowly absorbed than any of the other three sulphapyrimidines.

The blood concentrations attained by the drug following intraperitoneal and subcutaneous administration of 250 mg./kg. to mice are shown in Table I. From these it must be concluded that the drug is more rapidly and more completely absorbed by these routes than when given orally. Persistence in the blood is again marked, indeed there is no significant difference in this respect between the intraperitoneal and oral routes. The mean maximum blood concentration

of individual intraperitoneal curves (20.7 mg./100 ml.) is decisively higher than that of individual oral curves (15.3 mg./100 ml.), and the difference between the mean times of attaining these maximum values is also decisive. Owing to the difficulty of drawing some of the individual subcutaneous curves, no statistical comparison has been attempted in this case.

Sulphadimethoxypyrimidine can be detected in the blood of mice for several days after the administration of a single oral dose of 250 mg./kg. Blood concentrations recorded in a typical experiment with three mice were:

Time (hr.)	2	22	27	42	46	66	71	73	89	139
mg./100 ml.	13.2	13.85	13.4	5.0	5.3	4.0	4.2	1.2	0.25	0.14

The maintenance of high concentrations for so extended a time suggested that repeated dosing might have a cumulative effect, giving dangerously high concentrations. This possibility was examined by administering two oral doses of 250 mg./kg. at an interval of

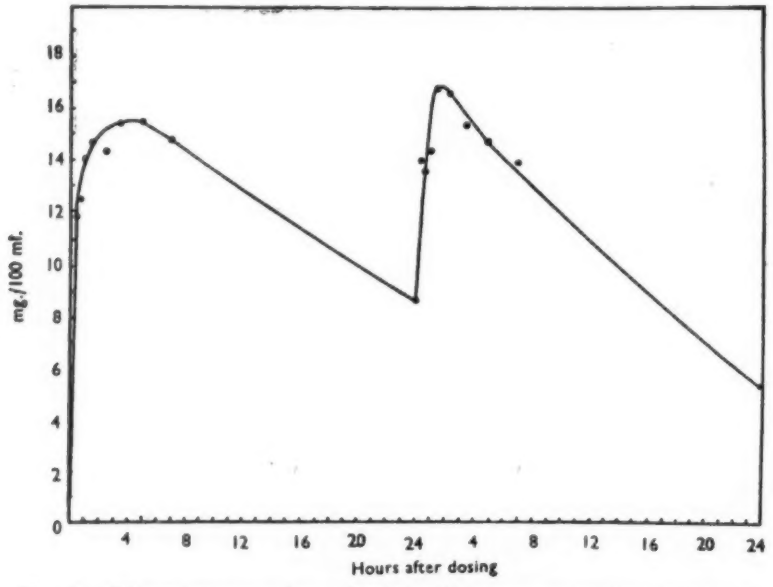


FIG. 2.—Blood concentrations of sulphadimethoxypyrimidine following the administration to mice of two oral doses of 250 mg./kg. at an interval of 24 hours.

24 hours to a group of three mice, and estimating the concentration of free drug in the blood at suitable intervals after each dose. The experiment was repeated ten times, with the results shown in Fig. 2. Clearly there is no marked rise in maximum blood level on such repeated dosing. This has been confirmed by statistical treatment, which shows that there is no significant difference between the mean maximum concentrations, but there are decisive differences between the mean times at which these maxima were attained and between the respective rates of disappearance of the drug from the blood stream. We have no explanation to offer for the greater rapidity with which the second dose is absorbed. but it may be that this phenomenon is associated in some way with the lower persistence.

Tissue concentrations in mice have been estimated following the usual dose of 250 mg./kg., three mice being used for each point recorded in Table II. In Table III the corresponding values for sulphamezathine are given for comparison; these have not been previously recorded. With both drugs, the concentrations in tissues approximated to those in blood in magnitude and persistence. Figures showing tissue distribution in the nephrectomized cat are given in the following section.

TABLE II

TISSUE CONCENTRATIONS OF SULPHADIMETHOXYPYRIMIDINE

250 mg./kg. orally in groups of 3 mice

Tissue	Concentrations in mg./100 g. of wet tissue after								
	30 min.	2 hrs.	4 hrs.	6½ hrs.	19 hrs.	31 hrs.	48 hrs.	72 hrs.	96 hrs.
Lung	14.5	16.9	19.6	17.6	16.3	10.1	2.6	3.8	2.1
Liver	15.1	13.6	12.9	15.0	14.6	4.0	2.7	2.5	1.1
Kidney	14.1	12.9	12.2	7.9	8.9	6.7	2.7	2.6	0.5
Spleen	7.0	6.8	9.5	9.5	10.8	5.5	1.6	0.5	0.6
Fat..		9.8							

The urinary excretion of the drug in the mouse has been examined in two groups of three animals. Following the administration of 250 mg./kg. orally, 46 and 32 per cent of the administered drug was excreted over three days in the two groups, and of these amounts 39 and 44 per cent respectively were acetylated. These results confirm the conclusion drawn from estimations of blood concentration after intraperitoneal injection, namely, that the drug is poorly absorbed in the mouse. The observation that, although much more persistent than sulphamezathine or sulphamerazine, sulphadimethoxypyrimidine gives lower blood concentrations than either of these compounds, can be explained on the same basis.

Experiments to determine the extent of conjugation of the new drug in the blood stream of the mouse are described below in the section dealing with acetylsulphadimethoxypyrimidine.

TABLE III

TISSUE CONCENTRATIONS OF SULPHAMEZATHINE

250 mg./kg. orally in groups of 3 mice

Tissue	Concentrations in mg./100 g. of wet tissue after				
	30 min.	2 hrs.	4 hrs.	7 hrs.	24 hrs.
Lung	21.8	17.1	12.3	6.5	2.3
Liver	18.0	18.8	10.7	3.1	3.0
Kidney	19.9	20.8	17.3	13.2	1.6
Spleen	14.8	11.1	6.3	4.2	1.4
Fat	10.7	6.6	7.1	5.3	1.9

In species other than the mouse.—The blood concentration-time curves presented have in each case been chosen as typical from at least three available in the particular species. The drug was given orally as a solution of the sodium salt in the following amounts: dog, calf, sheep, 100 mg./kg.; rabbit, 150 mg./kg.; rat, chick, 200 mg./kg. The

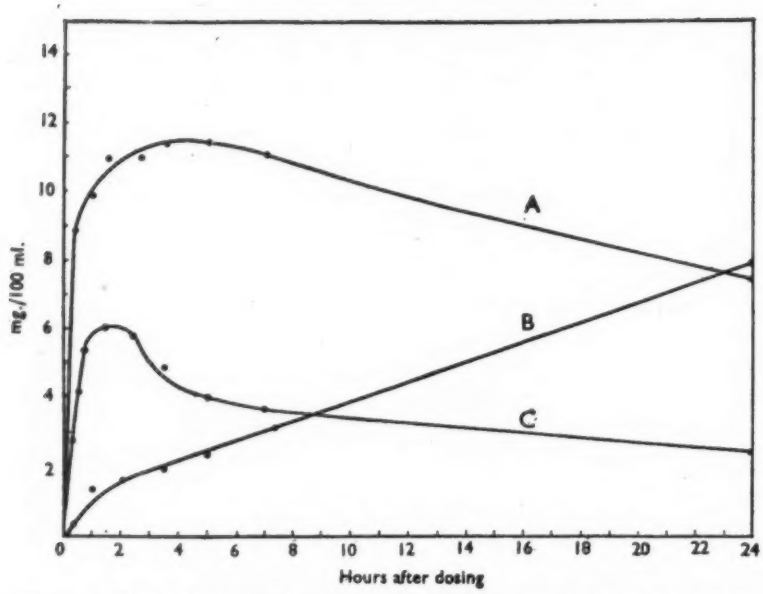


FIG. 3.—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 200 mg./kg. to the rat (A) and chick (B) and of 150 mg./kg. to the rabbit (C).

resultant blood concentrations are given in Figs. 3 and 4. In the rabbit, the urinary excretion of the drug has been measured, and the amounts appearing as free amine and in conjugated form estimated (Table IV). Rabbit A, which received 150 mg./kg. of the drug, excreted approximately 80 per cent in the urine, of which some 47 per cent was conjugated.

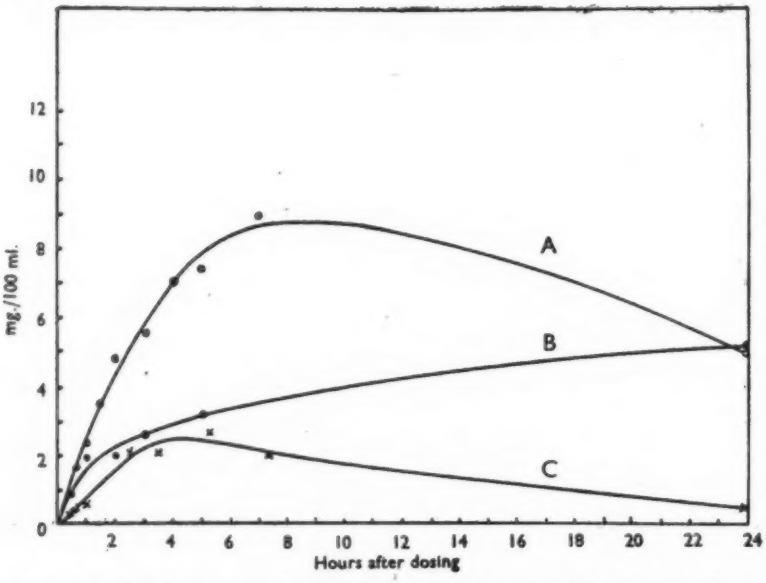


FIG. 4.—Blood concentrations of sulphadimethoxypyrimidine following the oral administration of 100 mg./kg. to sheep (A), calf (B) and dog (C).

TABLE IV

EXCRETION OF FREE AND CONJUGATED SULPHADIMETHOXYPYRIMIDINE IN THE URINE OF TWO RABBITS

Rabbit A (1.2 kg.; dose 180 mg.)					Rabbit B (2.17 kg.; dose 217 mg.)				
Day	Free drug		Total drug		Day	Free drug		Total drug	
	mg.	% of dose	mg.	% of dose		mg.	% of dose	mg.	% of dose
1	62.7	34.8	120.3	66.8	1	35.5	16.3	134.9	62.1
2	5.7	3.2	13.8	7.7	2	5.4	2.5	14.8	6.8
3	5.8	3.2	6.4	3.6	3	7.6	3.5	13.7	6.3
4 + 5	3.1	1.7	5.9	3.3	4 + 5	2.2	1.0	4.3	2.0
Total	77.3	42.9	146.4	81.4	Total	50.7	23.3	167.7	77.2

The proportion of total drug excreted by rabbit B, which was given a smaller dose of 100 mg./kg., was similar, but the percentage conjugated was much higher (70 per cent). Despite the high recovery, indicative of efficient absorption from the gastro-intestinal tract, the maximum blood concentrations attained in the rabbit were appreciably lower than those reached in the rat and mouse although a similarly high degree of persistence was observed. The expression C7, obtained from Fig. 3, had a value of 14 hours for the rabbit, as against 17 hours for the rat and 16.5 hours for the mouse. In the sheep, calf, and chick the drug was slowly absorbed but persisted well, particularly in the last two. Absorption was poor in the dog and removal from the blood stream rapid (C7=2 hours). Conjugation in rabbit and sheep is described in the section dealing with acetylsulphadimethoxypyrimidine.

The tissue distribution of the drug in nephrectomized cats has been estimated using the technique of Fisher, Troast, Waterhouse, and Shannon (1943). The results recorded in Table V are the means of three experiments and show the tissue/plasma ratio; they are compared with the figures obtained by Fisher *et al.* for sulphamerazine and sulphadiazine.

TABLE V

DISTRIBUTION OF SULPHADIMETHOXYPYRIMIDINE IN THE BILATERALLY NEPHRECTOMIZED CAT

Drug	Tissue/plasma concentration ratios							Vol. of distribution (as % of body weight)
	C.S.F.	Brain	R.B.C.	Lung	Liver	Pancreas	Muscle	
Sulphadimethoxypyrimidine	0.23	0.23	0.12	0.40	0.68	0.35	0.24	39
Sulphamerazine*	0.38	0.35	0.45	0.56	0.76	0.47	0.39	45.8
Sulphamezathine*	0.31	0.21	0.53	0.60	0.63	0.44	0.45	82.5

*Data of Fisher *et al.* (1943)

It will be seen that sulphadimethoxypyrimidine diffuses rather less into most tissues than the other sulphapyrimidines, and markedly less into the red blood cells; this may possibly be connected with the high protein binding of the drug (*vide infra*).

Antibacterial action in vitro

Comparisons of the antibacterial activities of sulphadimethoxypyrimidine, sulphamezathine and sulphadiazine were made by the method of Harper and Cawston

(1945). The medium used was Wright's broth containing 10 per cent (v/v) of lysed horse blood. Serial two-fold dilutions of the various sulphonamides in this medium were placed in 3 in. × ½ in. tubes in 0.5 ml. amounts, and 0.5 ml. of a 0.2×10^{-6} dilution in plain broth of a 24-hour culture of *Streptococcus pyogenes*, Kruger strain, was added to each tube. (This inoculum gave a count of approximately 5×10^{6} colonies per ml. on blood-agar.) The final

TABLE VI

COMPARATIVE ANTIBACTERIAL ACTIVITIES. RESULTS OF 48-HOUR PLATING ON BLOOD-AGAR

Organism: *Streptococcus pyogenes*

Sulphonamide	Concentration—one part in				Control
	40,000	80,000	160,000	320,000	
Sulphadimethoxypyrimidine	−	−	+	+	+
Sulphamezathine	−	±	+	+	
Sulphadiazine	−	−	±	+	

medium thus contained 5 per cent of lysed horse blood and the concentration of the sulphonamides ranged from 1 : 40,000 to 1 : 320,000. All tubes were incubated at 37° C. for 48 hours. One loopful from each tube was removed and plated upon blood-agar. The end-points were quite sharp and were recorded as − (no growth), ± (partial growth), and + (growth equal to control). The results are shown in Table VI. It will be seen that sulphadimethoxypyrimidine was intermediate in activity between the other two compounds.

Acute toxicity

When a suspension of sulphadimethoxypyrimidine was given orally to a group of 6 mice at the rate of 10 g./kg. none died. The intravenous injection of solutions of the sodium salts of sulphamezathine and of sulphadimethoxypyrimidine gave the results shown in Table VII, which is a summary of two experiments. All doses were contained in a volume of 0.2 ml., and each injection took 1 min. to complete.

TABLE VII

THE TOXICITY OF SULPHAMEZATHINE AND SULPHADIMETHOXYPYRIMIDINE BY INTRAVENOUS INJECTION OF THEIR SODIUM SALTS

Sixteen mice in each group. All mice observed for 5 days

Dose	Sulphamezathine	Sulphadimethoxy-pyrimidine
1.0 g./kg.	16 died	16 died
0.9 „ „	16 „	13 „
0.8 „ „	13 „	12 „
0.7 „ „	2 „	10 „
0.6 „ „	2 „	4 „
0.5 „ „	0 „	0 „

Chronic toxicity

Two separate experiments were carried out in which sulphadimethoxypyrimidine was administered as an aqueous dispersion once a day to rats for 28 days, at the rate of 1 g. per kg. body weight per day. The total number of rats in the two experiments was 54.

At the end of the period of administration of the drug the average final body weight of the treated animals was 130 per cent of their initial weight (from 110 g. to 143 g.), whilst the corresponding figure for equal-sized groups of matched control rats was 150 per cent.

Thirteen of the treated animals died during the period of treatment, or were killed because they were losing weight and were obviously ill, usually about 14 days from the start of the experiment. In several of these rats death was undoubtedly hastened by septic broncho-pneumonia brought on by maladministration of the doses of drug, but there were other deaths among rats whose lungs appeared normal. In the majority of the latter death could be ascribed to severe central necrosis of the liver lobules, sometimes affecting the whole lobule. It should be noted that the livers of 36 of the rats showed no abnormalities. Anaemia was very marked in many of the survivors, 5 of them giving readings of 29–46 per cent haemoglobin by the Sahli method. The spleens of many of the animals showed evidence of increased destruction of red blood corpuscles.

Although damage to the kidneys had been expected on account of the low solubility of this compound, such damage was seen in only 7 of the 54 animals examined. In only one of these was it severe, consisting of massive destruction of many convoluted tubules, amounting often to complete disintegration of the cell, with loss of nuclear staining and desquamation of the cells into the lumen of the tubule.

Therapeutic activity

Therapeutic experiments were carried out in mice infected with either *Streptococcus pyogenes*, Kruger strain (Group A), *Streptococcus pneumoniae* Type I, or *Staphylococcus aureus*. The first two organisms were given intraperitoneally and the third intravenously. All drugs were administered by mouth as aqueous solutions or dispersions, the desired dose

TABLE VIII

THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH *Streptococcus pyogenes*, *Streptococcus pneumoniae* OR *Staphylococcus aureus*

Oral doses of 100 mg./kg. twice daily for 3 days

Drug	*Streptococcus pyogenes*		*Streptococcus pneumoniae*		*Staphylococcus aureus*	
	No. of deaths in 7 days	Mean survival time in days (max. 7)	No. of deaths in 7 days	Mean survival time in days (max. 7)	No. of deaths in 14 days	Mean survival time in days (max. 14)
None (controls)	12	0.8	12	0.8	12	1.6
Sulphanilamide	12	1.0	12	1.2	10	5.0
Sulphapyridine	11	1.7	12	2.8	9	6.1
Sulphathiazole	12	1.0	12	2.1	8	7.2
Sulphamezathine	12	2.3	10	3.6	5	9.4
Sulphadiazine	11	3.8	12	4.3	4	11.2
Sulphamerazine	12	4.0	—	—	—	—
Sulphadimethoxy-pyrimidine	11	4.3	8	5.6	5	10.7

Streptococcus pyogenes. Infecting dose 0.2 ml. of a 1 : 10^3 dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth; given intraperitoneally.
Streptococcus pneumoniae. Infecting dose 0.2 ml. of a 1 : 10^3 dilution of an 18-hour culture (freshly isolated from a mouse) in 5 per cent (v/v) blood-broth; given intraperitoneally.
Staphylococcus aureus. Infecting dose 0.2 ml. of a 1 : 2 dilution of a plain broth culture, 18 hours old; given intravenously.

being contained in a volume of 0.5 ml. The drugs were administered shortly before the infection and further doses were given 7, 24, 31, 48, 55, 72, and 79 hours after the infection. The dose chosen for all the drugs in the first experiment (100 mg./kg.) was selected because experience had shown that, with streptococcal and pneumococcal infections, it permitted the various compounds to be ranged in order of activity on the basis of mean survival times. Table VIII summarizes the results of these experiments.

Taking all the experiments together, it will be seen that the increasing order of effectiveness of these compounds is approximately that in which they are arranged in the table.

In order to simulate more closely the conditions under which these drugs are used in clinical practice, another experiment was carried out in which infection of the mice preceded drug treatment by seven hours ; in this experiment the mice were infected intraperitoneally with streptococci. The results are shown in Table IX.

The results of this experiment range the drugs in substantially the same order as before. In both experiments sulphadimethoxypyrimidine compares very favourably with any of the other drugs.

TABLE IX

THERAPEUTIC RESULTS IN GROUPS OF 12 MICE INFECTED WITH *Streptococcus pyogenes*

Infected at 10 a.m. and treated at 5 p.m. of the same day with 500 mg. drug/kg. No further doses given

Drug	No. of deaths in group	Mean survival time in days (maximum 7)
None (controls)	12	0.8
Sulphanilamide	12	0.8
Sulphapyridine	12	1.5
Sulphathiazole	12	1.0
Sulphamezathine	12	1.5
Sulphadiazine	12	2.3
Sulphamerazine..	12	2.5
Sulphadimethoxypyrimidine ..	12	2.8

The infecting dose was 0.2 ml. of a 1 : 10^6 dilution of a culture of *Streptococcus pyogenes* prepared as in Table VIII.

Acetylsulphadimethoxypyrimidine

Estimation and occurrence.—As indicated above, sulphadimethoxypyrimidine appeared in a conjugated form in the urine of experimental animals. Since hydrolysis gave a diazotizable amine, the conjugated product was assumed to be the acetyl derivative and all estimations were made against this compound as standard. The method of Rose and Bevan (1944) proved satisfactory for the estimation of acetylsulphadimethoxypyrimidine in urine, but it yielded low recoveries when known amounts of the compound were added to blood and tissue homogenisates. Variable recoveries of the order of 60 per cent were obtained from human blood and 30 per cent or less from mouse tissues. The recovery from blood was increased to 70 per cent by hydrolysis of the conjugated drug in whole blood before the precipitation of proteins. This could not be regarded as satisfactory, and further work

showed that adequate recoveries were obtained from blood and tissue by diluting to 1 : 150 before precipitating protein. A suitable aliquot was then taken, hydrolysed with dilute hydrochloric acid, diazotized and coupled with N-β-sulphatoethyl-*m*-toluidine. A coupling time of 30 min. was required ; this is greater than that of the parent compound and probably indicates breakdown of the pyrimidine ring (cf. Frisk, 1943).

Estimations of free and total drug in the blood of mice receiving 250 mg. of sulphadimethoxypyrimidine/kg. orally, indicated the presence of traces only of conjugated amine. In the rabbit, on the other hand, high concentrations of conjugated drug were reached (Fig. 5). Similar results were obtained in the sheep, it being clear in both species that the acetyl derivative is similar in persistence to the free drug.

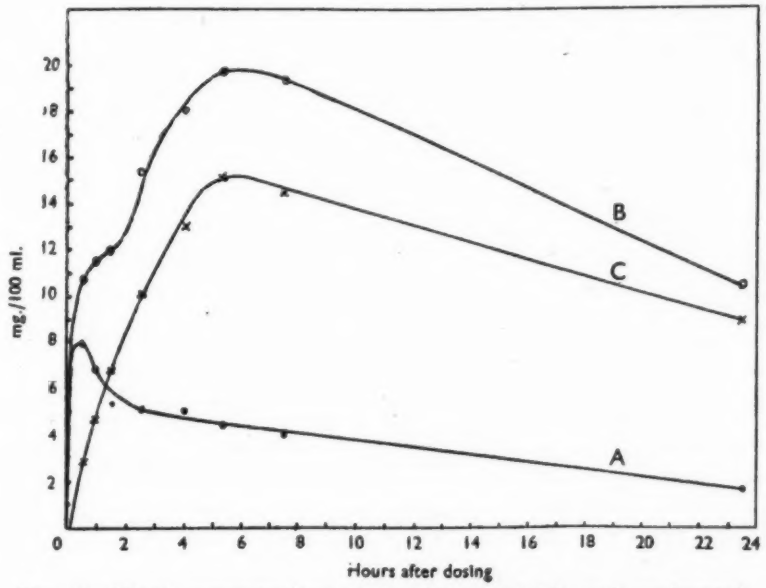


FIG. 5.—Blood concentrations of free (A), total (B), and conjugated (C, by difference) sulphadimethoxypyrimidine in the rabbit following the oral administration of 250 mg./kg. orally.

The administration of acetylsulphadimethoxypyrimidine was examined in the mouse. Four groups of three mice received 250 mg./kg. orally as a 1 per cent solution of the sodium salt. The mean blood concentration-time curves of free and total drug are shown in Fig. 6. That the observed hydrolysis of the acetyl derivative proceeds systemically and not in the lumen of the gut was readily shown by administering it intraperitoneally, when curves very similar to those of Fig. 6 were obtained. This facile hydrolysis of acetylsulphadimethoxypyrimidine undoubtedly accounts for its high activity, observed in the therapeutic experiments described below. In experiments with acetylsulphanilamide we found only traces of free drug in the blood following its administration in doses of 250 mg./kg., a result in agreement with its inactivity in therapeutic tests.

Therapeutic activity.—Only activity against *Streptococcus pyogenes* in mice has been examined in this instance, the infecting inoculum being similar to that used in the experiments recorded in Table VIII. The dosage regime was slightly altered in that amounts of 250 mg./kg. mouse (as against 100 mg./kg. mouse of the free amine) were given twice daily for three days, beginning shortly before infection. Acetyl derivatives of other sulphonamide drugs were included for comparison. Two series of experiments were made: in one

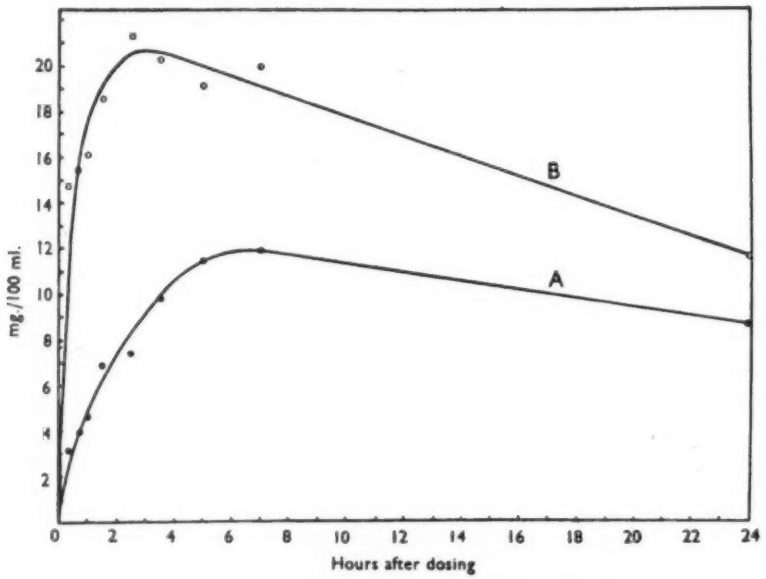


FIG. 6.—Blood concentrations of free (A) and total (B) sulphadimethoxypyrimidine following the oral administration in mice of 250 mg. of the acetyl derivative/kg.

the drugs were administered by mouth, and in the other intraperitoneally. The average survival times are indicated in Table X. Groups of 6 mice only were employed in each experiment.

Since the order of relative effectiveness of the acetyl derivatives is the same by both routes of administration, it appears that in all cases they are largely absorbed as such, and

TABLE X

THERAPEUTIC RESULTS OBTAINED IN GROUPS OF 6 MICE INFECTED WITH *Streptococcus pyogenes* 250 mg./kg. twice daily for 3 days

Drug	Oral		Intraperitoneal	
	No. of deaths in 7 days	Mean survival time (max. 7)	No. of deaths in 7 days	Mean survival time (max. 7)
None (controls)	6	0.8	6	0.8
Acetylsulphanilamide ..	6	0.9	6	0.8
Acetylsulphapyridine ..	6	0.9	6	0.8
Acetylsulphadiazine	6	1.1	6	1.0
Acetylsulphathiazole ..	4	3.2	6	2.3
Acetylsulphamezathine ..	6	1.5	6	1.0
Acetylsulphamerazine ..	6	0.8	—	—
Acetylsulphadimethoxypyrimidine	6	5.0	4	5.0

The infecting dose was 0.2 ml. of a 1 : 10^4 dilution of a broth culture of *Streptococcus pyogenes* prepared as in Table VIII.

absorption is not preceded by extensive hydrolysis to the free amines in the gastro-intestinal tract. This accords with the view expressed above in the case of acetylsulphadimethoxypyrimidine following direct estimation of blood concentrations of the free amine.

Physico-chemical properties

Solubility.—The solubility determinations were made in water at 37° C. by the method of Rose, Martin, and Bevan (1943), increasing *p*H by the addition of sodium hydroxide solution. The concentration of drug in a sample withdrawn through a filter plug into a pipette was determined colorimetrically. With the acetyl derivative, de-acetylation was necessary before the colorimetric estimation, which depended upon a diazo reaction, could be made. The solubility curves so obtained are shown in Fig. 7. In the *p*H range 6.0–7.0, the solubility of the free amine closely resembles that of sulphadiazine (Rose, Martin, and Bevan, 1943); thereafter the curve rises more steeply. Acetylsulphadimethoxypyrimidine differs from acetylsulphadiazine, however, in that it is less soluble than the parent amine in the *p*H range 6.0–7.3, but above the latter limit the acetyl derivative exhibits the greater solubility.

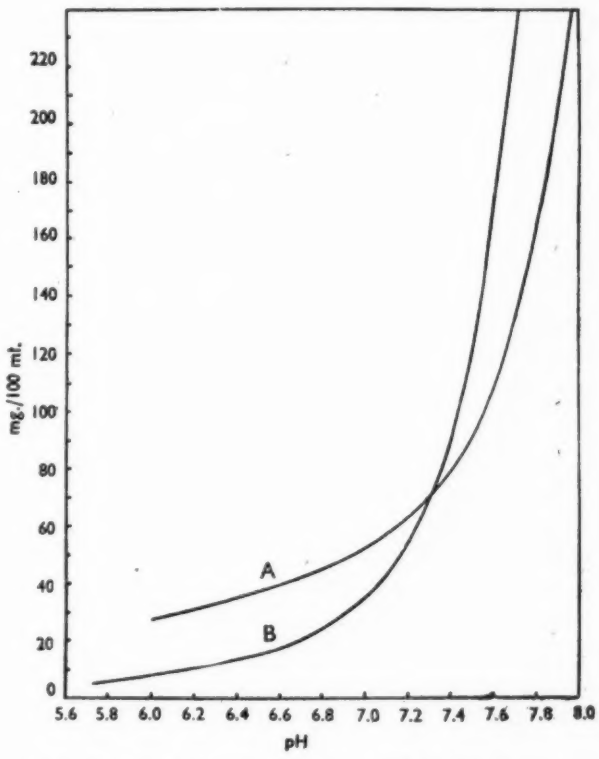


FIG. 7.—Solubility in water of sulphadimethoxypyrimidine (A) and of the acetyl derivative (B).

The acid dissociation constant.—The acid dissociation constant has been measured by potentiometric titration of the saturated aqueous solution and

TABLE XI

PROTEIN BINDING OF SULPHADIMETHOXYPYRIMIDINE

Plasma or serum	Percentage of drug bound to protein	
	Ultrafiltration	Dialysis
Rat plasma	85	82
Rat serum	87	80
Human plasma	—	80
Mouse plasma	—	78
Cat plasma	79	—
Sheep serum	63	—
Rabbit serum	71.5	—
Horse serum	63	—

the pK_a value found to be 7.00. A solvent partition method gave the value 7.02. The pK_a values for other sulphanilamide derivatives of pyrimidine are known—e.g., sulphadiazine 6.48; sulphamerazine 7.06; sulphamezathine 7.37 (Bell and Roblin, 1942).

Protein binding.—The association of the drug with blood proteins has been estimated by the equilibrium dialysis method of Davis (1943) and by ultrafiltration through collodion. Table XI shows the results obtained with various species.

It appears that sulphadimethoxypyrimidine is among the more highly bound sulphonamides, though a precise comparison is not possible, owing to the wide variation in values quoted for other drugs in the literature. The results are of the same order as the figure of 78 per cent quoted by van Dyke *et al.* (1945) for the same compound.

Summary and Conclusions

1. The properties of 2-*p*-aminobenzenesulphonamido-4:6-dimethoxypyrimidine (sulphadimethoxypyrimidine) are described, and include water solubility data for the free amine and the acetyl derivative over a range of *p*H.

2. The drug is relatively non-toxic in mice and rats, rather more slowly absorbed than sulphadiazine, sulphamerazine, or sulphamezathine when given orally to mice, but markedly more persistent in the blood-stream than these three drugs. Absorption data are given for other experimental animals.

3. A high percentage of the absorbed drug is excreted by the mouse in conjugated form; but feeding the acetyl derivative (or injecting intraperitoneally) gives rise, after a few hours, to a concentration of the free amine in the blood almost equal to that attained by initial administration in the latter form.

4. The antibacterial activity *in vitro* of the drug against *Streptococcus pyogenes* is intermediate between that of sulphamezathine and sulphadiazine, but therapeutic activity against this organism in the mouse is, in general, at least equal to or slightly better than that shown by any of the other sulphapyrimidine derivatives, in conformity with the high persistence of the drug.

5. Acetylsulphadimethoxypyrimidine given orally or intraperitoneally to infected mice exerts an appreciable therapeutic effect, greater than that shown by the acetyl derivatives of the several other heterocyclic sulphonamides examined.

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2-*p*-AMINOBENZENESULPHONAMIDO-4 : 6-DIMETHOXYPYRIMIDINE ABSORPTION AND EXCRETION IN MAN

BY

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WITH NOTES ON A CLINICAL TRIAL IN PNEUMONIA

BY

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(Received January 1, 1947)

The work of Gage, Martin, Rose, Spinks, and Tuey (1947) showed that a new sulphonamide, 2-*p*-aminobenzenesulphonamido-4 : 6-dimethoxypyrimidine or sulphadimethoxypyrimidine, had an unusual persistence when administered orally to animals. This fact, in conjunction with the observation that the antibacterial action *in vitro* and *in vivo* was of the same order as that of sulphadiazine, suggested that the compound merited a clinical trial, which was carried out in the medical wards of Crumpsall Hospital, Manchester. It was hoped that a sulphonamide which persisted in the blood for longer periods than other sulphonamides might be effective in the treatment of pneumonia in a single dose, or at most in doses once or twice daily.

METHODS

Sulphadimethoxypyrimidine was first administered to essentially normal patients, mostly convalescing from surgical and skin conditions, in doses ranging from 0.5 to 5 g. These patients were, at the time of testing, in fairly good general health and had no febrile condition ; except in one patient (receiving 0.5 g.) renal function was normal. Blood concentrations and recoveries in the urine are illustrated in the Table and Fig. 1. Two patients only were used for each dose level. The drug was also administered to a series of 80 patients, mostly suffering from pneumonia. One or two of these patients had other conditions normally requiring sulphonamide therapy. The results for 30 of these patients are shown in Figs. 2, 3, and 4 ; those obtained from the rest of the patients were similar although several patients had not received the full course before they were transferred to other treatments, penicillin or sulphamezathine, on clinical grounds.

Blood and urine sulphonamide estimations were made by the method of Rose and Bevan (1944). Twenty-four-hour collections of urine were made without preservative. For the blood urea, protein, phosphatase, and bilirubin estimations the methods of King (1946), slightly modified, were used.

RESULTS

Normal subjects showed a steady increase in maximum blood concentration and persistence with increasing dosage. (Table, Fig. 1.) The maximum was

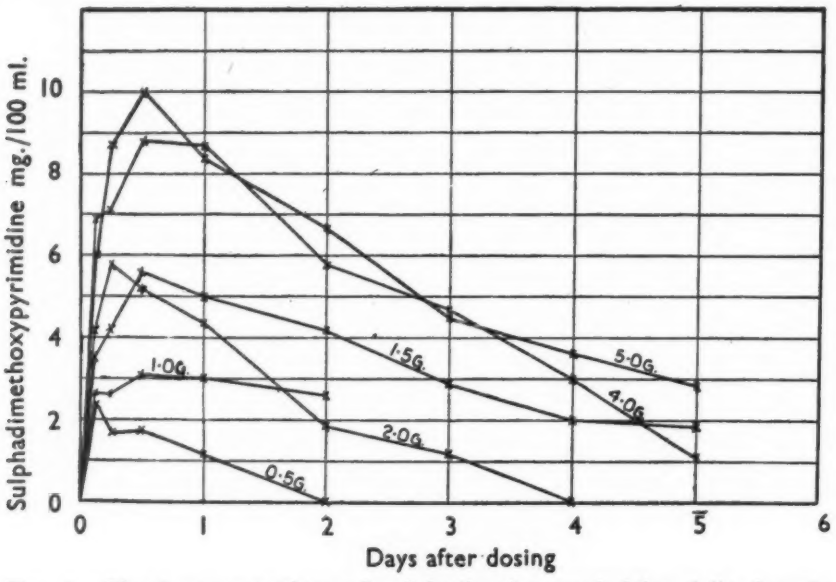

FIG. 1.—Blood concentrations of sulphadimethoxypyrimidine following the administration of single doses to normal subjects.

TABLE

NORMAL PATIENTS: MEAN BLOOD CONCENTRATIONS AND URINE RECOVERIES WITH SINGLE DOSES OF SULPHADIMETHOXYPYRIMIDINE

All readings are means for two patients (5.0 g. dose, one patient only)

Dose g.	Mean blood concentrations of free and, in parentheses, conjugated drug in mg./100 ml.										Total urine recoveries of free and, in parentheses, conjugated drug	
	1 hr.	2 hrs.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	2 days	3 days	4 days	5 days	mg.	Per cent of dose
0.5	1.25	2.05	2.45	1.7	1.75	1.2	Trace				74	14.8
	(0.1)	(0.05)	(0.2)	(0.05)	(1.25)	(0.55)	(0.85)				(18)	(3.6)
1.0	1.4	2.15	2.65	2.65	3.1	3.0	2.6	Trace			218	21.8
	(0.1)	(0.15)	(0.05)	(0.25)	(0.2)	(0)	(0.4)	(1.6)			(46)	(5.3)
1.5	0.4	1.85	3.5	4.2	5.65	5.05	4.2	2.9	2.05	1.9	500	33.3
	(0)	(1.05)	(1.0)	(0.95)	(0.7)	(0.85)	(0.7)	(0.2)	(0.35)	(0)	(77)	(4.4)
2.0	1.85	3.15	4.2	5.85	5.2	4.4	1.9	1.2	0		637	31.8
	(0)	(0)	(0)	(0.5)	(0.6)	(0.35)	(0.2)	(0.25)	(Trace)		(252)	(12.6)
4.0	1.15	4.05	6.0	8.75	10.05	8.45	6.7	4.55	3.65	2.85	835	30.6
	(0.25)	(0.1)	(0.25)	(0.65)	(0.45)	(0.6)	(0.4)	(0.45)	(0.25)	(0.2)	(241)	(6.0)
5.0	3.3	5.0	6.9	7.1	8.8	8.7	5.8	4.7	3.0	1.1	1202	24.0
	(0)	(0)	(0.3)	(0.1)	(0.2)	(0.3)	(0)	(0)	(0)	(0.5)	(415)	(8.3)

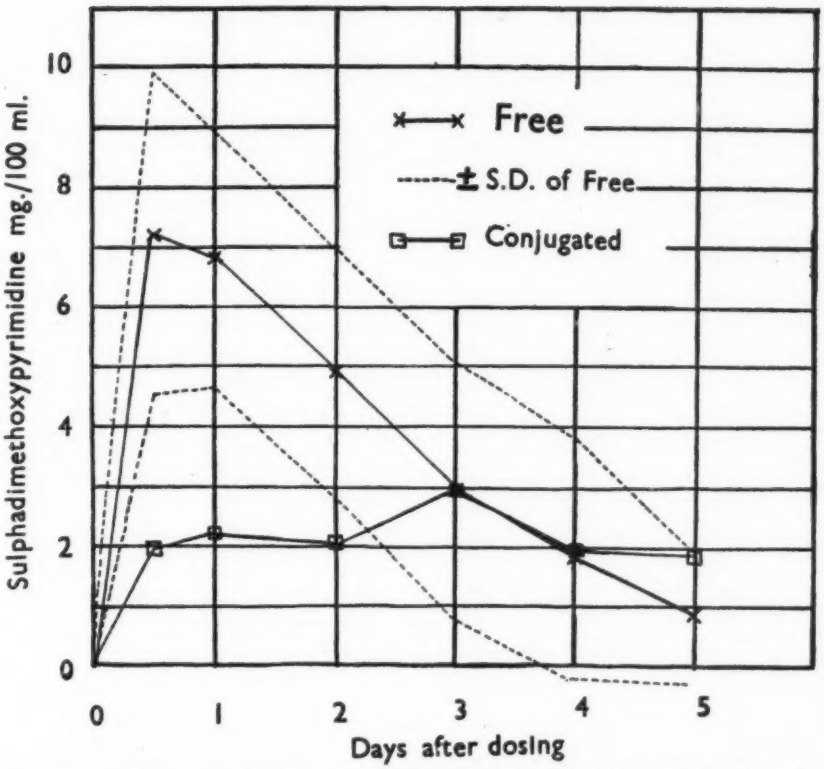

FIG. 2.—Blood concentrations of sulphadimethoxypyrimidine following the administration of a single dose of 5 g. to pneumonia patients.

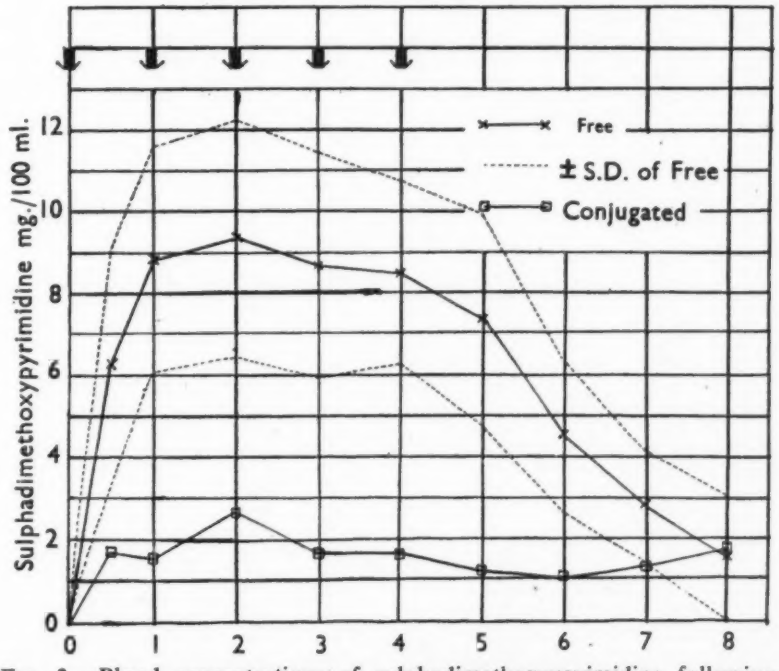

FIG. 3.—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g., and then 3 g., daily to pneumonia patients.

attained at three hours after a dose of 0.5 g., but with higher dosage the maximum was at 12 hours. After a single dose of 4 g. a blood concentration of 10.05 mg./100 ml. was attained after 12 hours and 2.85 mg./100 ml. persisted five days after the dose; a similar dose of sulphamezathine gave a maximum of 13.2 mg./100 ml., falling to traces within 24 hours. For patients suffering from pneumonia a single dose of sulphadimethoxypyrimidine gave on the average lower maxima and less well sustained blood concentrations (Fig. 2). The average maximum attained after 5 g. was 8.86 mg./100 ml., and only traces were detected in the blood after four days, although 2 mg./100 ml. were still present after three days.

In patients treated with 5 g. followed by 3 g. every 24 hours, blood concentrations were usually well maintained (Fig 3). In a few cases, however, the drug concentration did not reach a level of 8 mg./100 ml., a concentration usually regarded as effective chemotherapeutically. Patients with a low concentration of free drug usually showed a high percentage of acetylation, a frequent finding with other sulphonamides.

Since clinical results were not completely satisfactory it was decided to try a dose of 5 g. followed by 3 g. every 12 hours (Fig. 4). This dosage gave

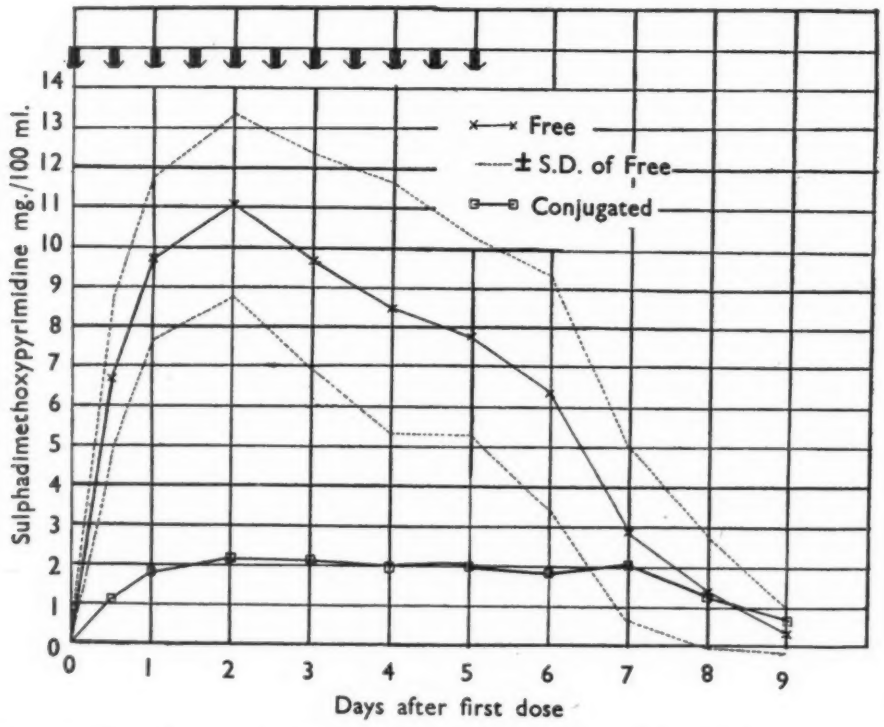

FIG. 4.—Blood concentrations of sulphadimethoxypyrimidine following the administration of an initial dose of 5 g., and then 3 g., at twelve-hourly intervals, to pneumonia patients.

slightly higher blood concentrations. All patients (except one who had only three doses) attained blood concentrations of over 8 mg./100 ml. In nearly all pneumonia patients receiving the drug, whether as single or repeated doses, free sulphadimethoxypyrimidine was still present in appreciable quantities three days after the last dose. In one patient with staphylococcal pneumonia and considerable renal damage (blood urea 100 to 150 mg./100 ml.), sulphonamide was still present in measurable quantities 16 days after the last dose, though the blood urea had fallen slightly.

Samples of cerebrospinal fluid were obtained from two patients and contained sulphadimethoxypyrimidine concentrations, in one case of 58 per cent, and in the other of 31 per cent, of the simultaneous blood concentration.

The blood concentration of sulphadimethoxypyrimidine reached a slightly lower maximum than did sulphamezathine after similar doses, but this maximum was attained later and high blood concentrations persisted for a very much longer period. There was a tendency for the concentrations reached in the controls to be higher than those attained with similar doses of the drug in febrile patients. Persistence of the blood concentrations after the last dose was definitely less in the febrile patients.

The recovery of the drug in the urine was comparatively low. The average recovery was 32.9 per cent, 26.6 per cent being free and 6.6 per cent conjugated in the normal patients, as compared with 86.2 per cent for sulphamezathine (Clark *et al.*, 1943), 68 per cent for sulphadiazine (Reinhold *et al.*, 1941), and 57.3 per cent for sulphapyridine (Long and Feinstone, 1938). The percentage of the drug recovered as acetyl compound, both from blood and urine, was low. These results must in part be interpreted in the light of the results of Gage *et al.* (1947) who record only partial recovery of the drug after acid hydrolysis.

Crystals of the drug were seen in many of the urines passed, but no renal symptoms were observed directly referable to the presence ot the crystals. In two patients with severe renal damage this damage could not be definitely assigned to the effect of sulphadimethoxypyrimidine. The crystals took the form of St. Andrew's crosses and were shown chemically to be free sulphadimethoxypyrimidine. When compared with the needle-shaped crystals often observed in the urine of patients receiving other sulphonamides, sulphadimethoxypyrimidine would not be expected to cause such extensive mechanical injury to the renal structures. Confirmatory evidence was provided by estimation of blood urea in 34 patients. The blood urea was usually about 40 mg./100 ml. on admission and in most cases tended to fall as the pneumonic condition improved. In no case was there a significant rise except in one woman dying within 48 hours of admission who was shown at autopsy to have severe chronic nephritis.

In chronic toxicity tests by Gage *et al.* (1947) large doses of sulphadimethoxypyrimidine in rats gave rise to severe central necrosis of the liver lobules, and it

was thought advisable to perform some liver function tests. Estimations of serum bilirubin, protein, and phosphatase were therefore made on nine patients. There was no change in the concentrations of these substances such as would indicate liver damage. It is realized that this series of tests would only show gross liver damage, and that minor impairment of function might pass unnoticed.

Summary

1. Sulphadimethoxypyrimidine in man persists in the blood stream longer than do the other common sulphonamides after similar doses.

2. Acetylation of the drug is slight.

3. Single daily doses of sulphadimethoxypyrimidine will give blood concentrations of an order considered adequate for therapy in pneumonia.

Clinical Trial

Sulphadimethoxypyrimidine was given to 41 patients with pneumococcal lobar pneumonia, whose ages varied between 16 and 78 years.

An initial dose of 3 to 5 g., followed at intervals of 24 hours by two doses of 2 g., was found to give a blood level of 5–13 mg. per 100 ml. which was maintained for 40 to 60 hours. Eighteen patients were treated in this way.

Nine patients were given an initial dose of 5 g., followed by 3 g. at intervals of 24 hours for four doses, a blood concentration of 5 to 14 mg. per 100 ml. being maintained for about 120 hours.

In thirteen patients an initial dose of 5 g. was followed at 12-hour intervals by two doses of 3 g. and then by 2 g. doses for several days, maintaining blood concentrations between 7–16 mg. per 100 ml. for four to six days. This method proved the best for maintaining adequate blood levels.

The drug was well tolerated and showed quite definite therapeutic value, but was not so effective as other sulphonamides used in series of similar cases (Don *et al.*, 1940 ; Macartney *et al.*, 1942 ; Ramsay *et al.*, 1945).

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AN ASSESSMENT OF THE VALUE OF SUGGESTED THERAPIES FOR LEUCOPENIA

BY

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(Received January 20, 1947)

The subcutaneous injection of methyl-bis(β-chloroethyl)amine (a nitrogen mustard) into rabbits consistently produces a leucopenia, and the degree of this leucopenia is readily varied by variation in the dosage of methyl-bis(β-chloroethyl)amine. This phenomenon has been used as a method of assessment of the value of three suggested therapies for leucopenia. The therapies tested were :

(i) Twenty-five per cent *p*-chloroxylenol in methylacetamide (CXM), which Zondek and Bromberg (1943) claim produces a leucocytosis lasting about four days in normal human subjects. In patients with typhoid fever the white cell count was maintained at a normal level by intramuscular injections of CXM, the count falling within 36 hours of discontinuing the injection.

(ii) The leucocytosis promoting factor (LPF) of Menkin and Kadish (1943). The latter state that 100 mg. of this preparation in saline or phosphate buffer at *p*H 7.4 when given to animals either by cardiac puncture or by subcutaneous injection causes leucocytosis.

(iii) 1 g./100 c.c. aqueous sodium succinate, 0.1 c.c. of which, when injected subcutaneously into normal subjects or phthisic patients, is said to raise the white cell count (Hammett, Vessler, and Browning, 1917).

Method of Assay

The leucopenia was produced by the subcutaneous injection of methyl-bis(β-chloroethyl)-amine hydrochloride solutions (1 mg./c.c.). Rabbits (circa 2 kg.) and occasionally goats (circa 30 kg.) were used as the test animals. The therapies under test were given at various intervals after the methyl-bis(β-chloroethyl)amine hydrochloride injections, the efficacy of the therapies being judged by daily white cell counts performed between 10 a.m. and noon and before feeding.

Results

(i) *CXM.*—This was given by intramuscular injection. Zondek and Bromberg's dosage for man (i.e., two doses of 10 c.c. the first day and 10 c.c. daily for the next three days) was given to goats. In normal goats only a slight rise in the white cell count was produced, while in methyl-bis(β-chloroethyl)amine hydrochloride-poisoned animals leucopenia was not prevented and the mortality was actually doubled (Table I).

A similar picture was obtained with rabbits. In normal rabbits a slight leucocytosis was produced with small doses, but raising the dosage caused all

TABLE I

EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF GOATS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu. mm. on days						
			Z–1	Z	1	2	3	4	5
8		20 c.c. day Z 10 c.c. days 2 and 3	7.0	7.0	9.6	10.4	10.1	8.7	7.7
4	1 mg./kg. day Z	—	9.9	11.0	10.0	3.0*	3.7	3.7	2.4*
4	1 mg./kg. day Z	20 c.c. day 1 10 c.c. days 3 and 4		11.0	5.4	7.8*	4.6	2.9*	—**

* = one animal dead.

the rabbits to die. With methyl-bis(β-chloroethyl)amine-poisoned rabbits leucopenia was not prevented and the mortality was increased (Table II).

TABLE II

EFFECT OF INTRAMUSCULAR CXM ON THE WHITE BLOOD CELL COUNTS OF RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu. mm. on days								
			Z–1	Z	1	2	3	4	5	6	8
4	—	0.5 c.c./kg. day Z	9.2	9.3	12.3	12.2	9.3*	6.3			
3	—	1 c.c./kg. day 1 5.0 c.c./kg. day Z	7.9	7.1	6.9*	6.2*	3.0*				
4	1 mg./kg. day Z	1 c.c./kg. day 1 0.5 c.c./kg. day 2		8.4	6.7	6.2*	1.2	2.4*	2.9	7.4	
5	1 mg./kg. day Z	5 c.c./kg. day 1		7.4	8.1	7.0	*** **				
5	1 mg./kg. day Z	2 c.c./kg. day 1 1 c.c./kg. day 2	7.4	7.5	7.4	6.8	6.5	4.4**	3.1	3.6**	
5	1 mg./kg. day Z	1 c.c./kg. days 1 and 2	6.5	7.0	6.5	6.4	5.8	4.9	3.4	3.4*	3.8
10	1 mg./kg. day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2

* = one animal dead.

(ii) *LPF.*—This was prepared from the inflammatory pleural exudates (produced by injection of turpentine) of rabbits and goats (LPF(R) and LPF(G)). When injected subcutaneously into normal rabbits or goats there was some evidence of the production of a slight leucocytosis, but with methyl-bis(β-chloro-

ethyl)amine hydrochloride-poisoned animals the leucopenia was not prevented and the death-rate was actually increased (Table III).

TABLE III

EFFECT OF LPF ON THE WHITE BLOOD CELL COUNTS OF GOATS AND RABBITS

Number of animals	Dosage nitrogen mustard	Therapy and dosage	Average white cell counts in thousands/cu. mm. on days								
			Z–1	Z	1	2	3	4	5	6	7
(a) RABBITS.											
5	—	100 mg. LPF(R) day Z and 10 mg. day 1	10.5	11.0	11.0	9.6	10.0	10.4			
4	—	100 mg. LPF(G) day Z	7.0	6.8	9.1	11.3	8.9	13.2	14.9	11.6	12.3
5	1 mg./kg. day Z	100 mg. LPF(R) day 1	9.6	9.7	9.6	4.8	4.6*	8.4**	8.2	10.9	10.0
3	1 mg./kg. day Z	100 mg. LPF(R) day 1 and 2		8.1	8.7	5.9	1.2	1.3	5.0**	10.2	
5	1 mg./kg. day Z	100 mg. LPF(G) day 1	9.1	10.2	9.6	8.9	9.2** *	9.8	13.0*	9.8	8.6
4	1 mg./kg. day Z	100 mg. LPF(G) day 1 and 2		7.5	7.0	3.9	1.0*	0.5	1.2	5.3	
10	1 mg./kg. day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2
(b) GOATS.											
2	—	300 mg. LPF(G) day Z and 1	7.3	5.6	12.9	11.5	10.8	9.0	9.8	8.4	
6	1 mg./kg. day Z	300 mg. LPF(G) day 1 and 2	8.1	7.9	14.9	9.9	7.3	5.5**	5.8**	5.4	
4	1 mg./kg. day Z	—	9.9	11.0	10.0	3.0*	3.7	3.7	2.4*	2.5	6.1

* = one animal dead.

(iii) *Sodium succinate.*—Various quantities and concentrations of sodium succinate in aqueous solution were injected subcutaneously into normal rabbits

TABLE IV

EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF NORMAL RABBITS

Number of animals	Therapy dosage	Average white cell counts in thousands/cu. mm. on days								
		Z–1	Z	1	2	3	4	5	6	7
5	0.1 c.c. 1% solution day Z	8.6	8.9	10.9	11.9	12.0	9.3	9.2		
5	0.5 c.c. 1% solution day Z	9.2	9.6	11.9	13.1	12.1	9.5	9.2		
5	1 c.c. 1% solution day Z	9.8	8.1	11.1	11.9	12.0	13.1	9.2		
5	1 c.c. 1% day Z; twice daily, days 1, 2, 3	9.1	9.8	11.8	14.2	20.8	10.8	14.2	15.0	9.8
5	1 c.c. 10% solution day Z		15.5	14.3	12.4	12.2	13.4	8.9	11.9	13.6

and there was definite evidence of the production of a leucocytosis, a 1 g./100 c.c. solution being better than a 10 g./100 c.c. concentration (Table IV). However, the sodium succinate solutions did not prevent the occurrence of leucopenia in methyl-bis(β-chloroethyl)amine hydrochloride-poisoned animals (Table V).

TABLE V

EFFECT OF SODIUM SUCCINATE ON THE WHITE BLOOD CELL COUNTS OF NITROGEN MUSTARD POISONED RABBITS

Number of animals	Dosage nitrogen mustard	Therapy dosage	Average white cell counts in thousands/cu. mm. on days								
			Z–1	Z	1	2	3	4	5	6	7
10	1 mg./kg. day Z	—	10.4	11.5	9.3	7.7*	6.3	5.4	4.8	8.3	9.2
15	1 mg./kg. day Z	1 c.c. 1% day 1	9.6	9.4	11.4	10.2	5.7	6.0*	10.2	10.5	
5	1 mg./kg. day Z	1 c.c. 1% at Z + 6 hours	—	12.0	13.9	10.2	6.9	6.6	12.7	—	—
10	1 mg./kg. day Z	0.5 c.c. 1% day 1	9.6	10.3	10.7	11.5	10.0*	11.3	11.8	12.0	10.6
5	1 mg./kg. day Z	1 c.c. 10% day 1	8.7	9.0	4.5	4.2	5.7	3.3**	—	—	—
10	2 mg./kg. day Z	—	10.5	14.0	12.5*	11.1*	9.2	3.9	11.7**	11.5	15.3
5	2 mg./kg. day Z	1 c.c. 1% days 1, 2, 3 and 4	13.2	13.6	14.9	16.7	6.7	10.4	16.8	12.9	—
10	2 mg./kg. day Z	1 c.c. 1% days 1–6	9.5	12.7	8.9*	6.9	5.1	2.7**	13.4*	11.9	7.9
5	2 mg./kg. day Z	5 c.c. 10% day 1	8.4	10.1	8.8	3.8	0.4	0.3*	3.7		
5	2 mg./kg. day Z	1 c.c. 1% at Z + 6 hours	9.2	8.4	14.5	4.2	0.9*	3.5**			

* = one animal dead.

SUMMARY

When assayed on rabbits or goats poisoned with methyl-bis(β-chloroethyl)-amine hydrochloride (given by subcutaneous injection), the following substances were ineffective in preventing leucopenia:

1. *p*-chloroxylenol in methylacetamide—this preparation, indeed, was found to be toxic in the recommended dosage.
2. The leucocytosis-promoting factor of Menkin.
3. An aqueous solution of sodium succinate.

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SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DI*ISO*PROPYL FLUOROPHOSPHONATE

BY

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The inhibition of cholinesterase by fluorophosphonates was discovered in 1941 by Adrian, Feldberg, and Kilby (1947), when they examined dimethyl fluorophosphonate. In 1941 McCombie and Saunders prepared di*iso*propyl fluorophosphonate and Adrian *et al.* (1942), as well as Mackworth (1942) found that it had an even stronger inhibitory action on cholinesterase than the dimethyl ester. At that time it was not known that there were two enzymes, true cholinesterase and pseudo-cholinesterase (Mendel and Rudney, 1943a), which are not necessarily affected similarly by inhibitors (Mendel and Rudney, 1944; Hawkins and Gunter, 1946). In the experiments to be reported in this paper it will be shown that di*iso*propyl fluorophosphonate, unlike eserine or prostigmine (Hawkins and Mendel, 1946, and unpublished experiments), exhibits a much stronger inhibitory action on pseudo-cholinesterase than on true cholinesterase. With low concentrations of di*iso*propyl fluorophosphonate it is therefore possible to inhibit pseudo-cholinesterase selectively without affecting true cholinesterase.

The possibility of such selective inhibition of pseudo-cholinesterase by di*iso*propyl fluorophosphonate was suggested by the following two observations:

(1) Bodansky (1945) as well as Mazur and Bodansky (1946) found that on exposure of human beings to low concentrations of the vapour of di*iso*propyl fluorophosphonate almost complete inhibition of cholinesterase activity in the plasma could be obtained without causing serious distress. It should be borne in mind, however, that human plasma contains predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943) and that the inhibition of this enzyme, as shown by Hawkins and Gunter (1946), will not interfere with the destruction of acetylcholine released by nervous activity. These workers found that certain concentrations of a prostigmine analogue, the dimethylcarbamate of 2-hydroxy-5-phenyl-benzyltrimethylammonium bromide (Hoffman-LaRoche Nu-683), are capable of inhibiting completely the activity of pseudo-cholinesterase *in vitro* without affecting significantly that of true cholinesterase. This

compound, when injected into dogs in amounts sufficient to inhibit pseudo-cholinesterase almost completely, elicits no symptoms indicative of the accumulation of acetylcholine ; such symptoms appear only if the dose injected is sufficiently large to depress the activity of the true cholinesterase as well. Pseudo-cholinesterase, therefore, plays no essential role in the hydrolysis of acetylcholine *in vivo*.

(2) Bodansky and Mazur (1946) and Mazur and Bodansky (1946) found that the concentration of di*iso*propyl fluorophosphonate necessary for the inhibition of cholinesterase varied according to the enzyme preparations used ; the negative logarithm of the molar concentration of di*iso*propyl fluorophosphonate necessary to produce a 50 per cent inhibition of the activity towards acetylcholine ($1.5 \times 10^{-2}M$) was 7.7 and 8.1 for human and horse serum respectively, the corresponding value for rabbit serum was 4.1, and the values for red blood cells and brain varied between 5.2 and 6.0. Since the sera of man and the horse contain predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943), rabbit serum mainly true cholinesterase (Mendel and Rudney, 1945), and brain and red blood cells throughout the animal kingdom true cholinesterase only (Mendel and Rudney, 1943a, 1943b), the results obtained by Mazur and Bodansky can be interpreted as indicating that pseudo-cholinesterase is approximately 100 times more sensitive to the inhibitory action of di*iso*propyl fluorophosphonate than is true cholinesterase.

Methods

Cholinesterase activity was measured manometrically by Warburg's method at 37° C. in $2.5 \times 10^{-2}M$ $NaHCO_3$, saturated with 5 per cent CO_2 in N_2. The di*iso*propyl fluorophosphonate was added to the bicarbonate medium containing the enzyme preparation in the main compartment of the Warburg flask, the substrate being placed in the side arm. After the enzyme preparation had been shaken for 15 min. to attain temperature equilibrium the substrate was tipped into the main compartment. From a stock solution, freshly prepared every third day, of di*iso*propyl fluorophosphonate ($10^{-2}M$) in propylene glycol, greater dilutions were made with distilled water as required. The final concentration of propylene glycol present in the experimental vessels caused by itself no inhibition of the cholinesterases.

The activities of the true and pseudo-cholinesterases were measured, as described by Mendel, Mundell, and Rudney (1943), by the rates of hydrolysis of acetyl-β-methylcholine and benzoylcholine respectively.

Results

In vitro.—Preliminary experiments were carried out to determine the inhibitory action of di*iso*propyl fluorophosphonate on enzyme preparations containing either only true cholinesterase or only pseudo-cholinesterase. This was done in order to ascertain whether the substrate acetylcholine could be replaced by acetyl-β-methylcholine in measuring the activity of true cholinesterase, or by benzoylcholine in measuring the activity of pseudo-cholinesterase, without affecting the percentage inhibition by di*iso*propyl fluorophosphonate. Haemolysed dog erythrocytes were used as a source of true cholinesterase, and an extract of dog pancreas as a source of pseudo-cholinesterase. As shown in Table I, the degree of

inhibition of true cholinesterase and pseudo-cholinesterase by di*iso*propyl fluorophosphonate is not altered when acetyl-β-methylcholine or benzoylcholine respectively are substituted for acetylcholine.

TABLE I

INHIBITION OF PSEUDO-CHOLINESTERASE AND TRUE CHOLINESTERASE BY DI*ISO*PROPYL FLUOROPHOSPHONATE, USING VARIOUS SUBSTRATES

Enzyme preparation	Substrate*	Molar concentration of di*iso*propyl fluorophosphonate	Percentage inhibition of enzymatic activity
True cholinesterase (haemolysed dog erythrocytes)	Ach. $1.2 \times 10^{-3}M$	1×10^{-7}	4
	Mch. $3 \times 10^{-2}M$	1×10^{-7}	3
	Ach. $1.2 \times 10^{-3}M$	5×10^{-7}	25
	Mch. $3 \times 10^{-2}M$	5×10^{-7}	26
Pseudo-cholinesterase (suspension of dog pancreas)	Ach. $6 \times 10^{-3}M$	1×10^{-8}	87
	Bch. $6 \times 10^{-3}M$	1×10^{-8}	86
	Ach. $6 \times 10^{-2}M$	5×10^{-8}	100
	Bch. $6 \times 10^{-3}M$	5×10^{-8}	100

* Ach. = acetylcholine; Mch. = acetyl-β-methylcholine; Bch. = benzoylcholine.

Moreover, the presence of pseudo-cholinesterase does not interfere with the inhibition of the true cholinesterase. Table II shows that the hydrolysis of acetyl-β-methylcholine by the true cholinesterase of haemolysed human erythrocytes is inhibited 26 per cent by $5 \times 10^{-8}M$ di*iso*propyl fluorophosphonate. When highly purified pseudo-cholinesterase prepared from horse serum is mixed with the haemolysed erythrocytes in an amount possessing an activity approximating that of the pseudo-cholinesterase of human plasma, no diminution of the inhibitory action of di*iso*propyl fluorophosphonate on true cholinesterase is observed.

In subsequent experiments, therefore, acetyl-β-methylcholine and benzoylcholine could be used to estimate separately the activities of the two cholinesterases in enzyme preparations which in most cases contained a mixture of both.

TABLE II

INHIBITORY ACTION OF DI*ISO*PROPYL FLUOROPHOSPHONATE ON TRUE CHOLINESTERASE IN THE PRESENCE OF PSEUDO-CHOLINESTERASE

Type of cholinesterase	Molar concentration of di*iso*propyl fluorophosphonate	Activity* (μl. CO_2/15 min.)	Percentage Inhibition
True cholinesterase (haemolysed human erythrocytes)	—	100.0	—
True cholinesterase (haemolysed human erythrocytes)	5×10^{-8}	74.0	26.0
True cholinesterase (haemolysed human erythrocytes) in the presence of pseudo-cholinesterase† (purified horse serum)	5×10^{-8}	74.5	25.5

* Substrate in all cases: acetyl-β-methylcholine ($3 \times 10^{-2}M$).

† The pseudo-cholinesterase from horse serum was kindly supplied by Miss F. Strelitz, who purified it according to her method (Strelitz, 1944). This preparation exhibited no activity towards acetyl-β-methylcholine.

The enzyme preparations tested were the plasma of man, dog, cat, rat, rabbit, and sheep. Sheep plasma contains no pseudo-cholinesterase, while the plasma of the other species contains both cholinesterases, although in different proportions. For each enzyme preparation, with the exception of sheep plasma, the minimal concentration of di*iso*propyl fluorophosphonate required to cause complete inhibition of pseudo-cholinesterase activity was determined, using benzoylcholine as substrate. The inhibitory action of this concentration of di*iso*propyl fluorophosphonate on the true cholinesterase in the plasma was then examined, using acetyl-β-methylcholine as substrate.

The results of these experiments are shown in Table III. Although pseudo-cholinesterase is inhibited completely in all instances, the true cholinesterase is inhibited only partially: 35, 34, and 33 per cent in human, rabbit, and rat plasma respectively, and only 7 per cent in dog plasma.

TABLE III

SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DI*iso*PROPYL FLUOROPHOSPHONATE

Source of enzyme	Substrate*	Molar concentration of di*iso*propyl fluorophosphonate	Activity (expressed as μl. CO_2 evolved by 1 ml. plasma in 15 min.) Without inhibitor	With inhibitor	Percentage inhibition
Human plasma	Ach. $6 \times 10^{-2} M$	1×10^{-6}	1280	13	99
	Mch. $3 \times 10^{-2} M$	,,	26	17	35
	Bch. $6 \times 10^{-3} M$	,,	570	0	100
Dog plasma	Ach. $6 \times 10^{-2} M$	5×10^{-6}	590	60	90
	Mch. $3 \times 10^{-2} M$	,,	82	76	7
	Bch. $6 \times 10^{-3} M$	,,	294	0	100
Cat plasma	Ach. $6 \times 10^{-2} M$	5×10^{-6}	426	14	96
	Mch. $3 \times 10^{-2} M$	,,	30	25	16
	Bch. $6 \times 10^{-3} M$	,,	109	0	100
Rat plasma	Ach. $6 \times 10^{-2} M$	1×10^{-6}	123	16	87
	Mch. $3 \times 10^{-2} M$	,,	54	36	33
	Bch. $6 \times 10^{-3} M$	,,	20	0	100
Rabbit plasma	Ach. $6 \times 10^{-2} M$	5×10^{-7}	45	20	56
	Mch. $3 \times 10^{-2} M$	,,	47	31	34
	Bch. $6 \times 10^{-3} M$	,,	4	0	100
Sheep plasma	Ach. $6 \times 10^{-2} M$	5×10^{-7}	13	10	23
	Mch. $3 \times 10^{-2} M$	,,	14	11	22
	Bch. $6 \times 10^{-3} M$	,,	0	0	—

* Ach. = acetylcholine
Mch. = acetyl-β-methylcholine
Bch. = benzoylcholine
} all in the form of the chloride.

Besides disclosing the difference between the sensitivities of pseudo-cholinesterase and true cholinesterase towards di*iso*propyl fluorophosphonate, these experiments show that when acetylcholine serves as substrate, the inhibition brought about by di*iso*propyl fluorophosphonate depends on the proportion of true cholinesterase and pseudo-cholinesterase present in the plasma; the greater the content of pseudo-cholinesterase, the greater the discrepancy between the inhibition of true cholinesterase and the inhibition observed when acetylcholine

is the substrate; conversely, the lower the pseudo-cholinesterase activity, the closer the parallelism between the inhibition of true cholinesterase and the inhibition of the acetylcholine hydrolysis.

In vivo.—Mazur and Bodansky found that in human beings exposed to di*iso*propyl fluorophosphonate, an inhibition of 98–99 per cent of the activity of the plasma towards acetylcholine did not result in symptoms of acetylcholine accumulation. In the present series, animals were injected intramuscularly with di*iso*propyl fluorophosphonate in order to ascertain whether in species, the plasma of which contains true cholinesterase and pseudo-cholinesterase in a proportion different from that in human plasma, there is also no correlation between the inhibition of the activity of the plasma towards acetylcholine and the symptoms to be expected from this inhibition.

Rabbits were chosen as experimental animals because the plasma of this species contains a smaller proportion of pseudo-cholinesterase to true cholinesterase, and therefore (see Table III) the discrepancy between the degree of inhibition of the activity towards acetylcholine, on the one hand, and towards acetyl-β-methylcholine, on the other, is not so pronounced as with human plasma, in which pseudo-cholinesterase predominates.

It will be seen from the typical experiment outlined in Table IV that rabbits receiving intramuscular injections of di*iso*propyl fluorophosphonate display their first symptoms of acetylcholine poisoning (i.e., masticatory movements of the jaws and slight generalized fibrillation) at a time when an appreciable activity (18 per cent) of the plasma towards acetylcholine is still present. These results confirm Mazur and Bodansky's findings in their experiments with rabbits. However, these authors did not attempt to explain why in rabbits symptoms of acetylcholine poisoning appear when the cholinesterase of their serum still displays a considerable activity towards acetylcholine, whereas in man an almost complete inhibition of the activity of the plasma towards acetylcholine causes no symptoms of serious distress.

TABLE IV

RELATIONSHIP BETWEEN THE INHIBITION OF CHOLINESTERASES BY DI*ISO*PROPYL FLUOROPHOSPHONATE AND THE ONSET OF SYMPTOMS OF ACETYLCHOLINE POISONING

Rabbit II—2.5 kg.
11.26 Activity of plasma tested.
11.27 0.65 mg. di*iso*propyl fluorophosphonate* in saline injected intramuscularly.
11.29 localized twitching of hind leg at site of injection.
11.40 masticatory movements, which continued until
11.49 0.13 mg. di*iso*propyl fluorophosphonate in saline intramuscularly.
11.56 generalized fibrillation.
11.57 chewing, swallowing and fibrillation; activity of plasma tested.

Time	Activity (expressed as μl. CO_2 evolved by 1 ml. plasma in 15 min.) towards:					
	Bch.† ($6 \times 10^{-3}M$)	Inhibition %	Mch.† ($3 \times 10^{-2}M$)	Inhibition %	Ach.† ($6 \times 10^{-2}M$)	Inhibition %
11.26	6.1	—	62.8	—	69.5	—
11.57	0	100	17.3	73	13.3	82

* An initial dilution (1 in 500) was made with propylene glycol.
† Bch. = benzoylcholine; Mch. = acetyl-β-methylcholine; Ach. = acetylcholine.

Discussion

The plasma of most species contains, in varying proportions, a mixture of two enzymes: pseudo-cholinesterase, which plays no essential role in the hydrolysis of acetylcholine *in vivo*, and true cholinesterase, the inhibition of which results in symptoms of acetylcholine poisoning. The experiments reported here have shown that appropriate concentrations of di*iso*propyl fluorophosphonate completely inhibit pseudo-cholinesterase without affecting the true cholinesterase significantly (see Table III).

Since acetylcholine is hydrolysed by both cholinesterases, measurements with acetylcholine as substrate can yield no information about the contribution made by each of these enzymes to the total activity, and the extent of inhibition of the activity towards acetylcholine in the presence of a selective inhibitor of pseudo-cholinesterase will depend on the relative proportions of pseudo- and true cholinesterases in the mixture which is being tested. Therefore, the degree of inhibition of acetylcholine hydrolysis by di*iso*propyl fluorophosphonate is no index of the inhibition of the true cholinesterase.

In the light of the above facts it is not surprising that human beings exposed to low concentrations of di*iso*propyl fluorophosphonate exhibit no symptoms indicative of acetylcholine accumulation when their plasma has lost 98–99 per cent of its original activity towards acetylcholine ; 99 per cent of the activity of human plasma towards acetylcholine ($6 \times 10^{-2}M$) is due to pseudo-cholinesterase, true cholinesterase accounting only for about 1 per cent of the total activity (Mendel, Mundell, and Rudney, 1943). Consequently, when di*iso*propyl fluorophosphonate causes a 98–99 per cent inhibition of the activity of human plasma towards acetylcholine, the inhibition of the pseudo-cholinesterase activity should be complete, while the activity of the true cholinesterase may be depressed less than 35 per cent (see Table III).

The results are entirely different with animals whose plasma contains predominantly true cholinesterase (e.g., rabbits). The hydrolysis of acetylcholine by the plasma of such animals is due mainly to the true cholinesterase ; therefore, when a 98 per cent inhibition of the activity of their plasma towards acetylcholine is achieved, it must be the true cholinesterase which is inhibited to a great extent. Consequently, symptoms of acetylcholine poisoning should set in at a much lower level of inhibition of acetylcholine hydrolysis than would be the case in species, such as man, where the hydrolysis of acetylcholine by the plasma is due mainly to pseudo-cholinesterase. Indeed, our experiments with rabbits have shown that the injection of di*iso*propyl fluorophosphonate leads to parasympathomimetic symptoms and fibrillation at a time when the activity of the plasma towards acetylcholine is inhibited not more than 80–82 per cent. In sheep, whose plasma contains true cholinesterase only, these symptoms would probably appear at a still lower level of inhibition of the acetylcholine hydrolysis. It would seem, therefore, that the higher the ratio of true cholinesterase to

pseudo-cholinesterase, the lower the degree of inhibition of acetylcholine hydrolysis prevailing at the time of onset of symptoms.

As mentioned previously, it is the inhibition of true cholinesterase which results in the appearance of symptoms of acetylcholine poisoning. On the basis of experiments in which the level of true cholinesterase activity was correlated with the appearance of symptoms after the injection of eserine, Gunter and Mendel (1945) concluded that the body possesses a surplus of this enzyme ; they observed no ill-effects until the activity of the true cholinesterase was inhibited 70–80 per cent. Similarly, Hawkins and Gunter (1946) found that symptoms of acetylcholine accumulation made their first appearance in dogs when the true cholinesterase activity of their plasma had been depressed to 23 per cent of its original level. Koelle and Gilman (1946) reported only slight parasympathomimetic symptoms in rats when the activity of the true cholinesterase in the brain had been depressed to 21–28 per cent of the normal by intramuscular injection of di*iso*propyl fluorophosphonate, and in the present study symptoms of acetylcholine accumulation appeared in rabbits when the activity of the true cholinesterase in the plasma had been depressed to 27 per cent of its original level (see Table IV).

Therefore, di*iso*propyl fluorophosphonate, in order to produce symptoms of acetylcholine poisoning, must be present in a concentration which is sufficient to remove the true cholinesterase in excess of that required for normal function. To estimate to what extent this objective has been achieved by injection of, or exposure to, di*iso*propyl fluorophosphonate it is necessary to determine the degree of inhibition of the activity towards acetyl-β-methylcholine. The use of acetylcholine as substrate would yield no such information except in the rare cases in which pseudo-cholinesterase is absent or is present in negligible amounts only.

Summary

1. Although di*iso*propyl fluorophosphonate inhibits both true and pseudo-cholinesterases, higher concentrations are required for the inhibition of true cholinesterase than of pseudo-cholinesterase. With appropriate concentrations of di*iso*propyl fluorophosphonate it is therefore possible, in a mixture of both enzymes, to inhibit selectively the activity of pseudo-cholinesterase without affecting that of true cholinesterase.

2. Acetylcholine is hydrolysed *in vitro* not only by true cholinesterase, but also by pseudo-cholinesterase ; therefore, measurements of cholinesterase activity in which acetylcholine is used as substrate cannot be used to correlate the degree of inhibition of true cholinesterase by di*iso*propyl fluorophosphonate and the effects resulting from this inhibition *in vivo*. Since true cholinesterase is the enzyme responsible for the hydrolysis of acetylcholine released at nerve endings, it is the degree of inhibition of true cholinesterase which must be determined

when a correlation between anti-cholinesterase action and pharmacological effects is sought.

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A PRELIMINARY REPORT OF THE TOXICITY AND THE ASSOCIATED BLOOD CONCENTRATIONS OF PALUDRINE* IN LABORATORY ANIMALS

BY

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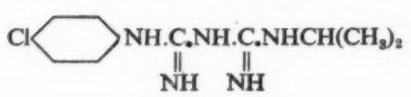
(Received February 25, 1947)

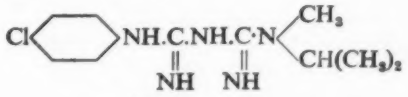
During the preparatory work before paludrine (4888) was administered to human beings the usual investigations were made of its toxicity in various laboratory animals. Early in the work we recognized that different species of animals behaved differently towards it; the rat and the mouse, for example, appeared more susceptible than the chick. We also recognized that the differences were possibly not due to a difference in susceptibility to unchanged drug, but more likely to a difference in its metabolism and the liberation of greater or less amounts of toxic by-products. Sufficient work was done to justify giving the drug to human beings, and the Liverpool workers (Adams, Maegraith, King, Townshend, Davey, and Havard, 1945) pursued their investigations on the assumption that human beings might react like the mouse and the rat, which are amongst the most susceptible of the laboratory animals. It was for this reason that, in the beginning, paludrine was given only twice daily, and doses were progressively increased by amounts not greater than 25 mg. Quite quickly it was shown that man must be classed amongst the least susceptible animals.

The laboratory results have not been published earlier because it was hoped that a fuller investigation would be made. Unfortunately, this will take longer than was planned, and because paludrine has now been sent to many laboratories in different parts of the world we are making the preliminary results available without further delay for the convenience of other workers.

The constitution of paludrine (base) is given below.



paludrine



4430

Two salts were used in the experiments, the monoacetate and the monohydrochloride. The former contains 81 per cent by weight of the base and is soluble

* Paludrine is the registered name for N_1-*p*-chlorophenyl-N_5-*iso*propylbiguanide.

to the extent of about 2 per cent in water; the latter contains 87.4 per cent by weight of the base and is about half as soluble. Solutions of either salt are stable when boiled. The figures quoted in the text, unless it is stated to the contrary, refer to the salts. The monoacetate was used in the intravenous and intraperitoneal tests, the monohydrochloride in the oral tests.

Acute toxicity

This was measured in the usual way. Solutions were administered orally by means of a catheter tube, and intravenously or intraperitoneally by rapid (3 sec.) injection. The results are given in Table I. For most species of animal three sets of figures are quoted which give, respectively, approximately the largest dose permitting all animals to live (LD0), the dose which kills approximately half the experimental animals (LD50) and approximately the smallest dose which kills all (LD100).

TABLE I

ACUTE TOXICITY OF PALUDRINE IN LABORATORY ANIMALS

Species	Route	LD0 (mg./kg.)	LD50 (mg./kg.)	LD100 (mg./kg.)
Chick (wt. 50 g.) ..	Oral	200	400–600	
	i.v.	40	60–80	100
Mouse (wt. 18–22 g.) ..	Oral	50	60–80	100
	i.v.	10	20–30	40–50
	i.p.	10	20–30	40–50
Rat (wt. 100 g.) ..	Oral	80	100–150	
	i.v.	20	40	60
	i.p.	20	40	60
Rabbit (wt. 1.5 kg.) ..	Oral		*circa* 150	
	i.v.	30	*circa* 50	

The intravenous or intraperitoneal injection of paludrine into both rats and mice is associated with delayed deaths, a point which is of much interest. It is best emphasized by comparing the results of an intravenous test using this drug with one using a closely related substance (4430), which differs only by a methyl group (see formula above). The results are given in Table II.

TABLE II

COMPARISON OF THE RESULTS FOLLOWING THE RAPID INTRAVENOUS INJECTION OF 4430 AND PALUDRINE INTO MICE

Dose	Results	
	4430	Paludrine
100 mg./kg. ..	6/6 mice dead within 3 min.	12/12 mice dead within 3 min.
80 mg./kg. ..	5/6 mice dead within 3 min; survivor alive 5 days later	9/18 mice dead within 3 min.; remaining 9 died 1 to 24 hours after the injection
60 mg./kg. ..	6/6 survived 5 days	No immediate deaths; 12/12 mice died 2 to 24 hours after the injection

At the time these experiments were carried out the blood concentrations of paludrine had not been measured, and it was thought possible that the delayed deaths were caused by unusually prolonged retention of the drug in the blood, and therefore that an additive effect might be produced by further intravenous injections.

The idea was tested by giving a second injection, after various intervals, of an amount (20 mg./kg.) that, by itself, produced very few deaths. Seventy-two mice were injected in the beginning; 12 were kept as controls and the remainder were divided into five further groups of 12 which were given a second injection 1, 3, 6, 24, and 48 hours respectively, after the first injection. The results are given in Table III. (The results of a second, similar experiment are given in parentheses in the Table.)

TABLE III

MORTALITY IN MICE AFTER A SECOND INTRAVENOUS INJECTION OF 20 MG./KG. PALUDRINE FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Figures in parentheses are the results of a second experiment

Group		Mortalities				Total dead after 5 days
		0–1 hr.	1–5 hr.	5–24 hr.	24–48 hr.	
I	Control ..	—	—	—	1/12 (1/12)	1/12 (1/12)
	2nd injection after					
II	1 hr.	—	1/12	9/12	2/12	12/12
III	3 hr.	—	—	9/12	—	9/12
IV	6 hr.	—	—	11/12 (6/12)	—	11/12 (6/12)
V	24 hr.	1/12	- -	4/12 (1/12)	2/12 (2/12)	7/12 (3/12)
VI	48 hr.	—	—	—	1/12	1/12

In mice, therefore, the second injection clearly exerts an additive effect. Similarly, a second injection of paludrine into rats also produces an additive toxic effect (Table IV), and in them, too, its parenteral injection is associated with delayed deaths. On the other hand, in chicks which have received paludrine intravenously, deaths occur within about 15 min. or not at all, and in them a second injection does not produce an additive effect.

TABLE IV

MORTALITY IN RATS AFTER A SECOND INTRAVENOUS INJECTION OF 25 MG./KG. PALUDRINE FOLLOWING A FIRST INJECTION OF THE SAME AMOUNT

Group		Mortalities					Total Deaths
		0–1 hr.	1–4 hr.	4–8 hr.	8–24 hr.	24–48 hr.	
I	Control	—	—	—	—	—	0/6
	2nd injection after						
II	1 hr.	2/6	—	1/6	3/6	—	6/6
III	3 hr. (1/6 dead before 2nd injection)	—	—	2/5	2/5	—	4/5
IV	6 hr.	—	—	2/6	3 6	—	5/6
V	24 hr.	—	—	—	—	—	0/6

Although these results tended to support the suggestion that paludrine might be highly persistent in rats and mice, measurements of blood concentrations soon disproved this, and we now find it difficult to believe that delayed deaths or additive effects are due to unchanged paludrine. We are therefore searching for a metabolite in the hope that the properties of the latter may provide an explanation.

Chronic toxicity

(*a*) *In mice.*—Two types of experiment were done. In the first, mice weighing 18 to 22 g. were arranged in groups of 10 and dosed twice daily for 5 days with the test solutions. The LD50 in this experiment is about 25 mg./kg.; at 12.5 mg./kg. no animals die; at 50 mg./kg. they all die.

In the second type, young mice weighing 14 to 16 g. were dosed twice daily for 14 days. Growth appeared normal amongst those receiving 12.5 mg./kg.; deaths occurred at higher doses.

(*b*) *In rats.*—Newly weaned rats, weighing about 40 g., and selected from as few litters as possible, were arranged in groups of 10. Sexes and litter mates were distributed equally among the various groups. Food (standard cubes made to a formula of the Rowett Institute) was given to the animals immediately after the daily weighing at 10 a.m.; water was always available.

The growth of rats for the first few weeks after weaning is linear and, with careful matching, all the groups in experiments such as the ones being described can be made to follow the same straight line. Seven days were allowed for the line to become established, and then treatment with paludrine was commenced. It was given orally, once daily.

It was found that a dose of 50 mg./kg. caused an immediate alteration in the slope of the growth curve, although not sufficient to reduce it to zero. Scattered deaths also occurred with this treatment. With a dose of 40 mg./kg. a slight deviation of the curve was caused sometimes immediately, sometimes later. With a dose of 30 mg./kg. growth was normal over the whole period of treatment (two months in some experiments) and there were no deaths.

Rats which died, and the survivors of all groups, were subjected to a pathological examination, but nothing of significance was found.* It is noteworthy that in none of these toxicity tests has any symptom been produced in any of the mammalian species that would lead one to suppose that the drug had affected the central nervous system. Chemical estimations confirm that the amount of drug which can be recovered from the brain of rats and rabbits is insignificant (see below and Spinks, 1947).

Blood concentrations†

The rat growth test just described is probably one of the most sensitive toxicity tests available in the laboratory and it was regarded as important to determine the concentrations of paludrine in the blood associated with the doses,

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*We are indebted to Dr. J. R. M. Innes for this information. The organs examined were brain, kidney, liver, pancreas, spleen, lung, intestine, and thyroid.

† All concentrations, whether in plasma, whole blood or tissue, are given as mg./l. or mg./kg. of the free base.

50 mg./kg. and 30 mg./kg. per day respectively, which delimited the toxic region. They were measured by the method of Spinks and Tottey (1946). Measurements on whole blood were made, in different experiments, after the first dose and after the seventh dose. At least three rats were used in the determination of each point. A curve for the concentrations reached on the seventh day is given in Fig. 1.

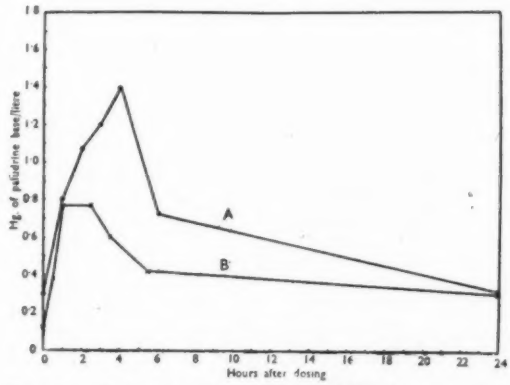

FIG. 1.—Concentrations of paludrine in the whole blood of rats after the seventh dose of 50 mg./kg. (A) and 30 mg./kg. (B) once daily.

The most interesting feature of the results is the fact that the blood concentration associated with a dose of 50 mg./kg. once daily, which produces deaths in some rats, is comparatively low and has a peak of only about 1.4 mg./l. That such a concentration should be toxic in rats is of interest, because we believe it to be readily tolerated by human beings; 700 mg. of paludrine have been administered twice daily in man with only mild toxic effects (Adams *et al.*, 1945), while plasma concentrations of about 0.5 mg./l. have been recorded twelve hours after doses of 500 mg. twice daily (Maegraith *et al.*, 1946). The maximal concentration in the plasma following the latter dose was found to be about 0.7 mg./l. in one subject, who, however, showed minimal concentrations rather lower than normal (Maegraith *et al.*, private communication). Since the whole blood concentration in man is between 2 and 3 times the plasma concentration (Maegraith *et al.*, 1946), it is reasonable to assume that blood concentrations between 1 mg. and 1.5 mg./l. are attained following the administration of 500 mg. twice daily. The comparison can also be made on the basis of plasma concentrations. The maximal plasma concentration given in rats by the (toxic) dose of 50 mg./kg. daily is 0.236 mg./l. (Table V). Concentrations much higher than this have been frequently observed in man. A further point of difference between man and rat is the lower persistence of paludrine in the latter, illustrated by the low minimal concentrations, and by the fact that the concentrations determined after only one dose are very similar to those determined after 7 doses.

It now became important to determine the blood concentrations associated with chronic toxic effects in other species. We chose to examine the mouse and the chick because the mouse behaved like the rat in the matter of delayed deaths after parenteral injection, whereas the chick did not. Curves for whole blood concentrations in these two species are given in Figs. 2 and 3.

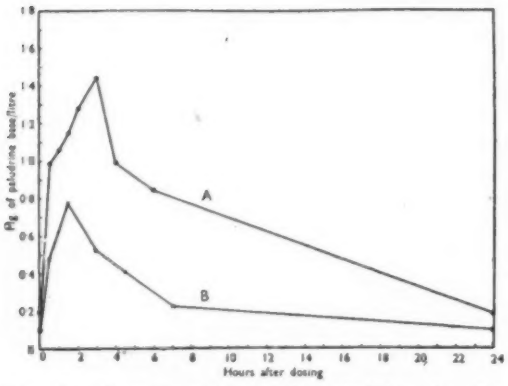

FIG. 2.—Concentrations of paludrine in the whole blood of mice after the third dose of 30 mg./kg. once daily (A) and the fifth dose of 12.5 mg./kg. twice daily (B).

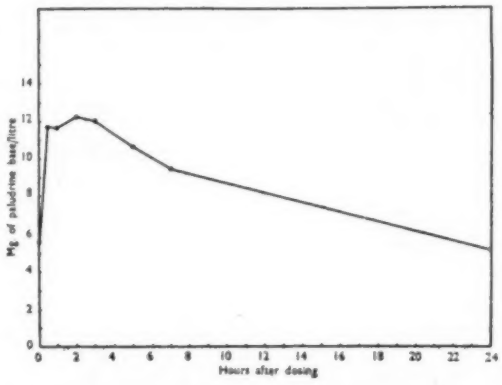

FIG. 3.—Concentrations of paludrine in the whole blood of chicks after the fourth dose of 60 mg./kg. twice daily.

The curves for mice were determined on one group receiving 12.5 mg./kg. twice daily, a treatment which is apparently harmless, and on another receiving 30 mg./kg. once daily, a treatment which produces scattered deaths. In the first group measurements were made after the fifth dose, in the second after the third dose. It will be seen that there is a good parallelism between the concentrations toxic for mice and those toxic for rats, and that the general form of the curves is similar, the build-up being negligible. It will also be apparent that the same dose given to mice and rats on a weight basis will produce higher concentrations in the mice.

Curves for chicks were obtained from animals receiving 60 mg./kg. twice daily. Scattered deaths occur with this regime although about 30 to 50 per cent of the animals will survive indefinitely treatment given for 5 days. Measurements were made after the fourth dose. It will be seen that the peak concentrations associated with a potential lethal effect in chicks are about 10 times as high as concentrations associated with lethal effects in rats and mice. Also the build-up is considerable, a residue of about 5 mg./l. being left after the third and fourth doses. The contrast between chicks on the one hand, and rats and mice on the other, is also well shown by the results of other experiments in which only one dose of 50 mg./kg. was given to chicks. Peak concentrations of paludrine in the blood rose to 3–4 mg./l. and after 24 hours concentrations of 1.5 mg./l. were recorded.

DISCUSSION

Clearly a fundamental difference must exist between the metabolism of paludrine in rats and mice and its metabolism in chicks (and probably man). Certainly, the grosser aspects of distribution which can be measured chemically do not account for the differences in results. In all species examined so far the concentration of the drug in the plasma is about a third to a fifth that in whole blood, and the ratios between tissue and plasma concentrations (which vary from 10 to 100/1 depending on the tissue) are similar. We sought to emphasize the distinction between the chick and the rat, and the fact that the tissue distribution of the drug does not reveal any explanation of it, by comparing the concentrations found in the tissues of rats and chicks following the seventh dose of

50 mg./kg. once daily. This treatment is lethal for some rats, but tolerated by chicks. The blood and tissues of 3 rats or 6 chicks were used at each time interval. The results are shown in Tables V and VI.

TABLE V

DISTRIBUTION OF PALUDRINE IN RATS FOLLOWING THE SEVENTH ORAL DOSE OF 50 MG./KG. ONCE DAILY

Time	mg. base/l. or kg. in						
	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain
Before ..	0.403	0.0693	1.61	0.414	0.577	0.748	0
1 hour ..	0.802	0.0804	4.34	2.62	3.03	20.6	0
2 hours ..	1.07	0.154	11.2	4.69	9.14	32.4	trace
4 „ ..	1.40	0.236	17.9	12.3	9.80	30.1	0
6 „ ..	0.723	0.144	6.41	1.95	2.10	11.9	trace
24 „ ..	0.320	0.0671	1.82	0.711	0.947	1.07	(0.123)

TABLE VI

DISTRIBUTION OF PALUDRINE IN CHICKS FOLLOWING THE SEVENTH ORAL DOSE OF 50 MG./KG. ONCE DAILY

Time	mg. base/l. or kg. in						
	Blood	Plasma	Lung	Spleen	Kidney	Liver	Brain
Before ..	3.25	1.02	72.4	25.9	92.6	45.3	11.5
1 hour ..	3.78	1.31	62.9	26.6	89.7	62.8	10.5
2 hours ..	8.15	2.84	121	73.8	214	136	19.4
4 „ ..	7.59	2.58	117	57.6	246	104	17.5
6 „ ..	5.91	2.14	103	40.9	177	95.1	18.7
24 „ ..	2.95	0.809	75.6	12.4	53.9	31.6	15.1

Although the treatment is tolerated by chicks, but fatal for some rats, the drug concentrations are uniformly higher in the chicks. It would seem, too, that paludrine reaches the brain more readily in the chick than in the rat, a point which is of interest because, so far as we are aware, such a species difference has not been demonstrated for any other drug. However, this difference would hardly seem to have any bearing on the high blood concentrations in the chick or the delayed deaths in mice and rats. Surveying the results of all the experiments, we have come to the conclusion that the simplest explanation of them is to postulate that paludrine, in mice and rats, is metabolized to a substance persistent in the body and more toxic than the drug itself; in chicks, and probably in man, the metabolism is either different qualitatively or, if it is similar, the degree of degradation to the toxic substance is much less. The relevant facts can be summarized as follows:

1. Equivalent doses of paludrine give higher concentrations, and the drug is more persistent, in chicks than in mice and rats. On the other hand, it is more

toxic for mice and rats than for chicks. On the evidence so far available, it is probable that man behaves like the chick rather than like the rat or mouse.

2. Although paludrine appears to be removed so readily from the blood of mice and rats, delayed deaths may occur in both species and, after parenteral administration, an additive toxic effect can be produced by a second injection given even 24 hours after the first.

3. The tissue distribution of the drug in its grosser aspects does not account for the differences in susceptibility between chicks and rats. Concentrations are uniformly higher in the chicks.

4. The distribution of 4430 (an N_5-methyl derivative of paludrine) in the body is similar to that of paludrine (Spinks, 1946, 1947), but delayed deaths are not associated with its injection into animals.

5. Recoveries of paludrine from the faeces and urine of rats (and rabbits) are low, usually less than 30 per cent of the dose (Spinks, 1947), which contrasts markedly with what obtains in man, where they are much higher, often up to 60 per cent (Maegraith *et al.*, 1946).

Summary

1. Measurements of the toxicity of paludrine for mice, rats, rabbits, and chicks are given.

2. Delayed deaths follow the intravenous injection of paludrine into mice and rats, and it is noteworthy that an additive toxic effect can be obtained by a second intravenous injection given many hours after the first. Delayed deaths do not follow the intravenous injection of the drug into chicks, and in these animals an additive toxic effect is not produced by a second injection.

3. Measurements of the concentration of paludrine in the blood of mice, rats, and chicks under various treatments showed that chicks tolerate much higher concentrations of the drug in the body than do mice and rats.

4. Gross measurements of the drug in the organs of chicks and rats on a similar treatment (50 mg./kg. once daily) did not reveal differences sufficient to account for the difference in tolerance. Because of this, and in the light of other evidence which is presented, it is suggested that the metabolism of paludrine in chicks is different from what it is in rats and mice. The facts would be explained if paludrine, in mice and rats, were degraded in large measure to a substance more toxic than the drug itself.

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pA, A NEW SCALE FOR THE MEASUREMENT OF DRUG ANTAGONISM

BY

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When the activity of a drug can be expressed in terms of a stable standard which does not differ from it qualitatively, there is no difficulty in getting reproducible results, since all the assays tend to give the same answer, and any degree of accuracy can be attained provided that the experiment is repeated sufficiently often. When, however, the activity of a new drug or drug antagonist has to be defined in terms either of some other drug or of some of its own effects, the results are not equally reproducible since the apparent activity varies in successive experiments, even though conditions are kept as constant as possible. The difficulty of making results of one laboratory available to another is aggravated by the multiplicity of methods used and frequently by the lack of information of their variability; this applies particularly to methods of expressing drug antagonism.

It would obviously be of advantage if some common method of expressing drug antagonism could be agreed upon. In the present paper it is proposed to introduce a new measure of drug antagonism, pA, based on a suggestion made originally by Clark and Raventos (1937). Apparatus and methods are described for determining pA accurately on the guinea-pig's ileum, and the activity of several known antagonists of histamine and acetylcholine has been measured in terms of pA. The variability of the pA measure has been estimated and methods are discussed of obtaining reproducible results in the most economical way.

Apparatus

All the experiments were done on the isolated ileum of the guinea-pig. The apparatus used for assaying drug antagonists is shown in Fig. 1. It consists essentially of a gut bath which can communicate with two alternative systems, one of which is filled with ordinary Tyrode solution and the other with Tyrode solution containing the antagonistic drug; the latter solution can be replaced in the course of the experiment without interfering with the assay.

All the operations involved in an assay, except the injection of the drug, are performed automatically. The principle of the method has been described before (Schild, 1946): "Telephone relays are converted to compress rubber tubing. When the relays are activated the rubber tubing is decompressed and fluid is allowed to flow. These relays control the emptying and filling and the adjustment of fluid level of an isolated organ bath. They are activated at regular time intervals through a telephone uniselector which makes 12 successive

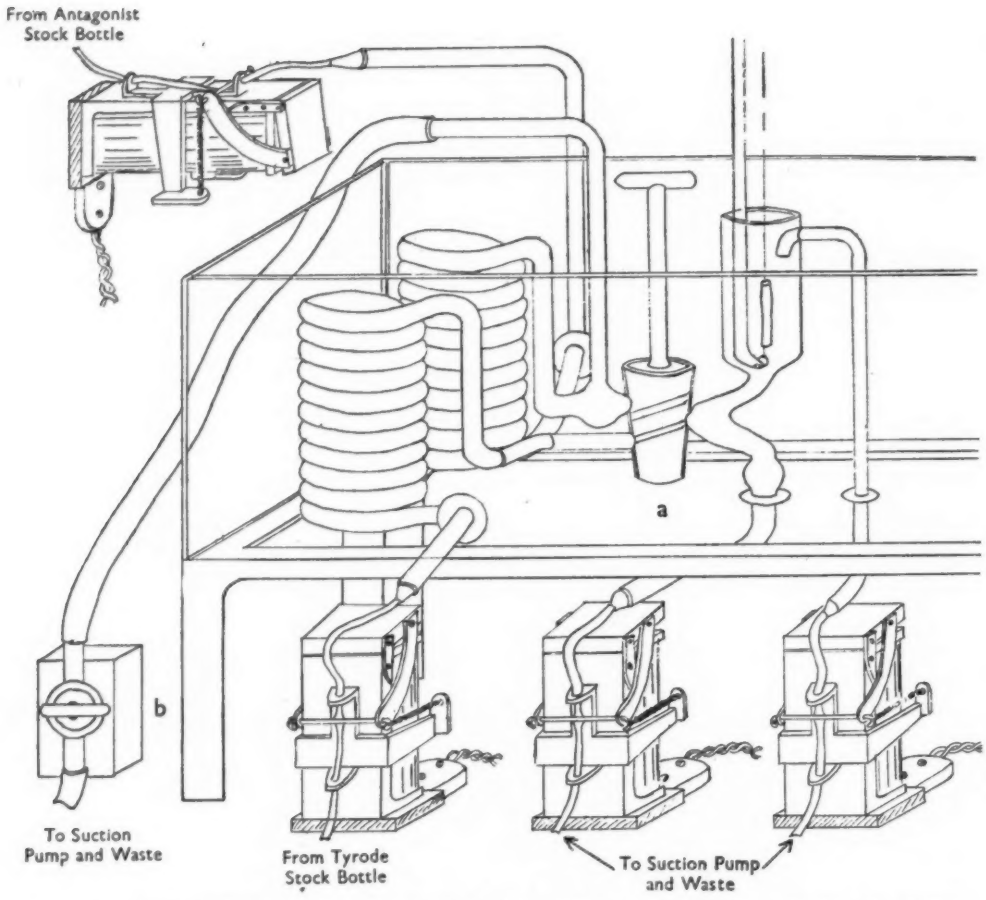

FIG. 1.—Apparatus for assaying drug antagonists. Two stock bottles provide the inflow; one filled with Tyrode solution, and the other with a Tyrode solution of the antagonist drug. All the outflows are connected to a water suction pump. The electro-magnets compressing rubber tubing are automatically operated through the selector circuit shown in Fig. 2. They are standard P.O. telephone relays of 3,000–6,000 ohms resistance operated directly from D.C. mains, with a special armature made in our workshop. The movement of the armature is controlled by two coiled springs which tend to compress rubber tubing of 2×4 mm. diameter by means of a thin brass rod.

contacts in a cycle. The duration of each contact is usually 15 sec., thus producing a cycle of 3 min. The selector also controls the movements of the drum and a light signal to time the injection of drugs." A diagram of the selector circuit is shown in Fig. 2.*

The present apparatus differs in the following respects from the one previously described:

1. An additional inflow relay is provided to control the inflow of the antagonistic solution. The two inflow relays are activated simultaneously, allowing one of the two solutions to flow into the bath according to the position of the 3-way tap. By means of

*All the compon parts of the automatic apparatus, including A.C. mains-operated electro-magnets comp g rubber tubing, and A.C.-operated 12-step selectors, are now made by Messrs. Londe , Ltd.

switch D (Fig. 2) the selector can be short-circuited and the inflow relays activated directly. If the solution in the system containing the antagonistic drug is to be replaced, the 3-way tap *a* (Fig. 1) is turned into the null position, switch D is activated, and tap *b* is opened, allowing the solution to drain into the exhaust.

2. Switches A, B, and C control certain alternative arrangements of the cycle. By means of switches A and B the gut bath may be emptied and refilled twice in succession instead of only once. By means of switch C the signal may be advanced by 15 sec., permitting a longer period of contact between drug and tissue. Table I shows the automatic operations performed in a complete cycle together with the possible alternative arrangements.

3. The outflows are operated by suction.

4. A new type of adjustment for telephone relays has been used to compress rubber tubing. The adjustment is somewhat more complicated than the one previously described, but it is more efficient and stable. It is illustrated in Fig. 1.

5. Two gut baths have been operated simultaneously. The baths were contained in a large thermostatically controlled tank, stirred by a circulating water-pump. As a rule a lag period of 30 sec. was maintained between the two cycles, tracings being recorded on two independent drums situated at opposite ends of the tank.

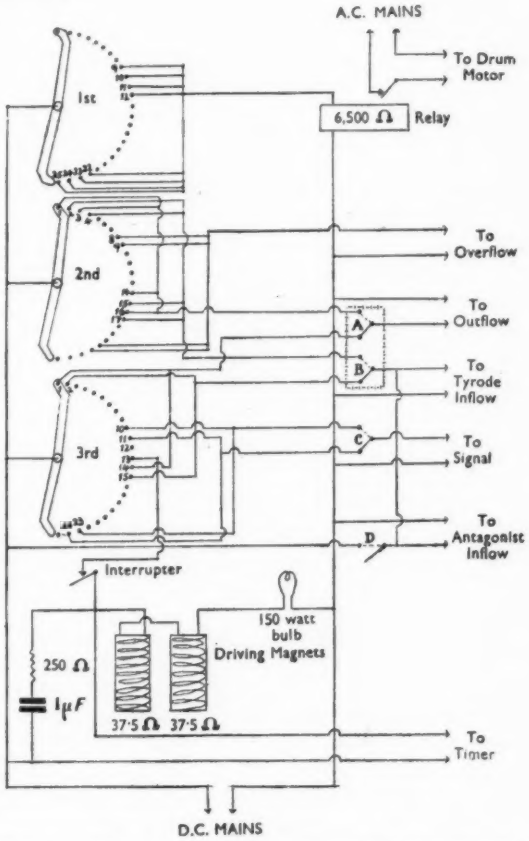

FIG. 2.—Diagram of selector circuit for one muscle bath.

TABLE I

STEPS IN CYCLE

Relays	1	2	3	4	5	6	7	8	9	10	11	12
Outflow { usual	+											
Outflow { alternative	+		+									
Inflow { usual		+										
Inflow { alternative		+		+								
Signal { usual											+	
Signal { alternative										+		
Overflow								+	+			
Drum									+	+	+	+

The writing lever used in these experiments is illustrated in Fig. 3.

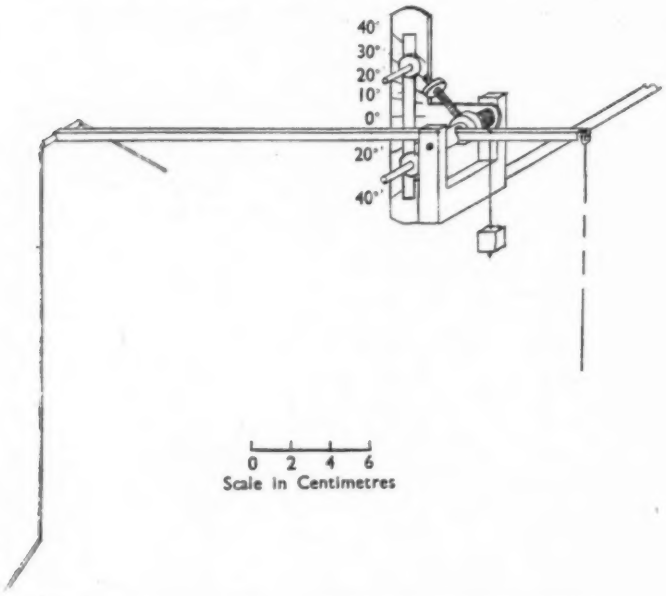

FIG. 3.—Approximately linear and isotonic frontal writing lever.

It may be asked to what extent these modifications of the usual Burn-Dale isolated organ bath are essential for these experiments.

The purpose of the automatic apparatus is to ensure constant time intervals and bath volumes, to enable more than one assay to be performed at the same time and in general to enable the experimenter to divert his attention from servicing the bath and drum. In practice the automatic apparatus has been found very useful, but it is obviously not essential for carrying out these tests since all the operations can be performed by hand.

The main purpose of adding the antagonistic drug to the bath fluid before it reaches the gut, instead of adding it in the usual way by injection into the muscle bath, is to prevent the gut coming into contact with pure Tyrode solution during the period of replacement of the bath fluid when the effect of an antagonist on successive contractions is being investigated. The present method is thus particularly suitable for investigating the effect of antagonists in relation to time, whilst both methods are suitable for determining the effect of antagonists on a single contraction. The old method is most suited for preliminary experiments and for comparative measurements.

The purpose of the long writing point used on the frontal writing lever is to ensure that the relation between shortening of the gut and effect on the drum should be linear. This is especially important when the effects are measured in terms of a maximum contraction. The errors obtained with a shorter writing point are, however, usually not excessive, unless a very short writing point is used or the angle of excursion of the lever becomes greater than about 30° from the horizontal; this may be prevented by means of two adjustable stops limiting the excursion of the lever as shown in Fig. 3. The errors may be calculated from the formula given in a previous communication (Schild, 1944). The lever can be made practically isotonic by means of the screw adjustment shown on the pivot. The adjustment may be tested by suspending an appropriate weight at the point of attachment of the thread. The lever should then balance in every position in which it is likely to be used. The tension exerted by the lever in these experiments was of 600–800 mg.

The following antagonist drugs were used in these experiments:

Neoantergan, or N-*p*-methoxybenzyl-N-dimethylaminoethyl-α-aminopyridine acid maleate (Bovet, Horclois, and Walthert, 1944).

Benadryl, or dimethylaminoethyl benzhydryl ether hydrochloride (Loew, Kaiser, and Moore, 1945).

Pethidine, or ethyl 4 - phenyl - 1 - methylpiperidine - 4 - carboxylate hydrochloride (Schaumann, 1940).

Atropine sulphate.

THE pA SCALE

pA_x is defined as the negative logarithm to base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose. Thus, if the presence of a concentration of $10^{-5.8}$ molar pethidine in the bath fluid reduces the effect of 2 μg. histamine to that produced, in the absence of pethidine, by 1 μg. histamine, pA_2 pethidine-histamine = 5.8.

It is obvious that a constant of this nature can refer only to a given drug-antagonist pair acting on a definite pharmacological preparation, e.g., the guinea-pig's ileum, and that a representative pA value must be the mean of several individual determinations. pA values are dependent on the length of contact between antagonistic drug and tissue, but they are apparently independent of the absolute concentrations of the active drug used. These points will be discussed in detail later.

Method of determining pA_2.—The principle of the method consists in finding two concentrations of the antagonistic drug such that one will reduce the effect of a double dose of the active drug to slightly less and the other to slightly more than the effect of a single dose. The concentration corresponding to pA_2 is then computed by interpolation on a logarithmic scale.

The following results justify to some extent the use of a logarithmic scale for interpolation. In a series of pA_2 determinations a third concentration of antagonist was added to test for linearity between log concentration of antagonist and effect. The points, plotted on a logarithmic scale in Fig. 4, each point representing the mean of several determinations, fall on approximately straight lines.

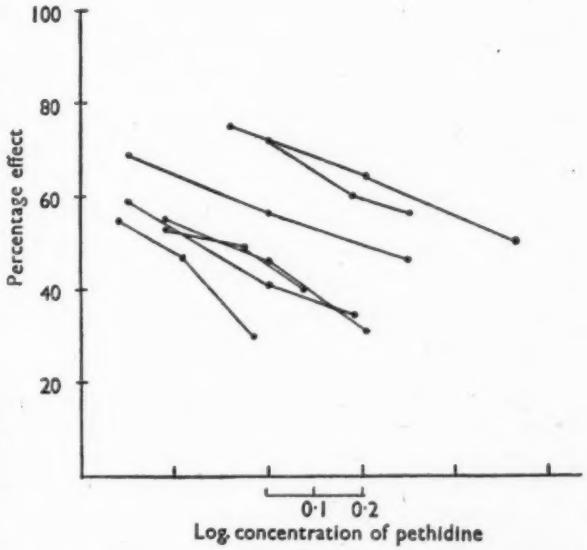

FIG. 4.—Guinea-pig ileum. Pethidine-histamine. Approximately linear relation between log concentration of pethidine and reduction of histamine effect.

In order to get reliable results a constant submaximal response to the stimulant drug must be produced before addition of the antagonist; 10–20 preliminary contractions may be required to achieve this. At this point the muscle chamber

is joined to the system containing a Tyrode solution of the antagonistic drug, which now replaces Tyrode solution when the bath fluid is changed. At the next injection the dose of active drug is doubled. Injections of a double dose of active drug in the presence of the antagonist are continued for several periods (usually five periods) in order to observe whether the effect of the antagonist increases with increasing length of contact. Eventually the bath fluid is switched back to Tyrode solution to test for persistence of antagonistic effect and the assay is concluded by producing a series of maximal effects. A second concentration of antagonist is investigated in the same way, usually on a fresh piece of gut, and the results, expressed in terms of the maximal contraction, are used for interpolating pA_2 values after various periods of contact with the antagonist.

As in these experiments the automatic apparatus was set to produce intervals of 3 min. between injections and pauses of 2 min. between completion of change of bath fluid and the next injection, the first pA measurement was made when the antagonistic drug had been in contact with the tissue for 2 min. and further determinations at 3 min. intervals. During this period the tissue never ceases to be in contact with the antagonistic drug which is contained in the bath fluid itself, and an even flow of stimulation at constant intervals is maintained as the period of contact with the antagonist is gradually increased.

Although tedious, the use of a fresh piece of gut for each concentration of the antagonist has been found to be the most satisfactory procedure when the effect of the antagonist is persistent. In our experience, variations in sensitivity to the action of antagonists of different segments of the same gut are remarkably small and are not correlated with variations in sensitivity to the stimulant drug, provided that pieces which are obviously damaged and insensitive are rejected. Although it is advantageous to use two pieces of gut simultaneously, this is not essential, since the guinea-pig ileum does not seem to alter appreciably in sensitivity if left in clear Tyrode solution at room temperature for several hours.

Fig. 5 (p. 205) illustrates two complete pA_2 determinations done on six segments of the same gut. The object was to determine pA_2 values after a short period of 2 min. and after a long period of 14 min. contact with the antagonist. The following two examples, quoted from the experiment shown in Fig. 5, illustrate the method of computing pA_2:

pA_2 benadryl-histamine (2 min. contact).—After 2 min. contact between muscle and antagonistic drug a double dose of histamine in $10^{-7.94}$ molar benadryl (1:300 million) produces an effect which is 7 per cent (of the maximal effect) greater, and a double dose of histamine in $10^{-7.46}$ molar benadryl (1:100 million) an effect 11 per cent smaller, than that produced by a single dose of histamine in Tyrode. By interpolation the molar concentration of benadryl which would just reduce the effect of a double dose of histamine to that of a single dose is $10^{-7.75}$ (1:195 million). Hence pA_2 (2 min. contact)=7.75.

pA_2 neoantergan-histamine (14 min. contact).—When the antagonist has been in contact with the muscle for 14 min. (5th injection in presence of antagonist) a double dose of

histamine in $10^{-9\cdot55}$ molar neoantergan (1:9,000 million) produces an effect 7 per cent greater, and the same dose in $10^{-9\cdot08}$ molar neoantergan (1:3,000 million) an effect 10 per cent smaller, than a single dose of histamine in Tyrode. By interpolation pA_2 (14 min. contact)=9.36.

Independence of pA and concentration of antagonist.—Since the absolute concentration of the active drug does not enter into the definition of pA, it was of interest to find out whether the depression produced by a certain concentration of the antagonist was, in fact, independent of the concentration of active drug used. Although this is generally accepted as true (Gaddum, 1937), it seemed worth investigating the point, using a properly randomized experimental design which could be statistically analysed.

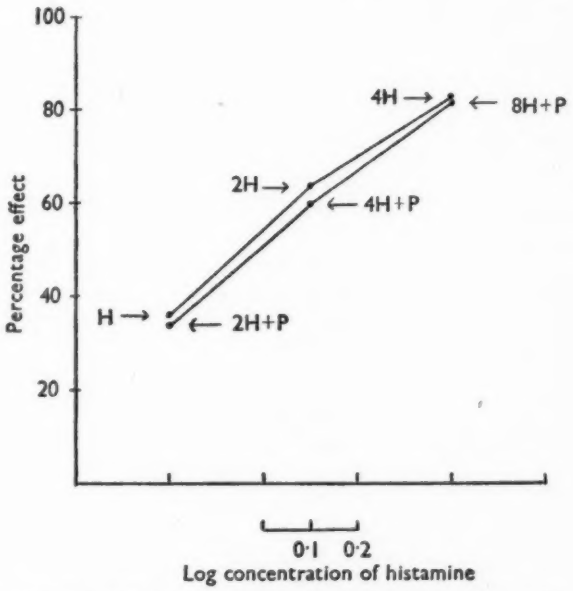

FIG. 6.—Effect of a constant dose of pethidine (P) on contraction produced by various concentrations of histamine (H). The depressor effect of the antagonist is independent of the contraction produced by the active drug. Upper curve: histamine alone. Lower curve: double dose of histamine+pethidine.

The experiments were carried out as follows. Several doses of the active drug alone and of the active drug plus antagonist were administered. If the antagonist produced the same amount of depression at each concentration of the active drug, the concentration–action curves in the presence and the absence of the antagonist should be parallel. Doses were administered repeatedly in a random order, and the results were analysed by means of analysis of variance for statistically significant deviations from parallelism.

Five such experiments were performed; three with pethidine-histamine, one with atropine-acetylcholine, and one with pethidine-acetylcholine. One of the experiments is illustrated in Fig. 6, each point on the curve representing the mean of four determinations. It will be seen that at each level the reduction of the histamine effect by pethidine was approximately the same. There was no statistically significant deviation from parallelism between the two curves. Similar results were obtained in the other experiments.

ANTAGONISM OF NEOANTERGAN, BENADRYL, PETHIDINE, AND ATROPINE TOWARDS HISTAMINE AND ACETYLCHOLINE

The activity and relative specificity of these antagonists of histamine and acetylcholine can be conveniently summarized in terms of pA. In Fig. 7 the

two vertical lines represent pA_2 scales for antagonists of histamine and acetylcholine. The activity of an antagonist towards these drugs is indicated by its position on the scale, one scale division corresponding to a tenfold difference in activity. Points on the two scales referring to the same antagonist are joined. If an antagonistic drug does not discriminate between two active drugs, the line joining the scales is horizontal; if it discriminates sharply the line is steep, as with neoantergan and atropine. Neoantergan is the most discriminating of the four antagonists, being 40,000 times as active against histamine as it is against acetylcholine. Atropine is 1,000 times as active against acetylcholine as it is against histamine. Pethidine, on the other hand, discriminates hardly at all between the two.

A single pA value is not sufficient to characterize an antagonist fully. To describe fully the relation between a given antagonist and an active drug it would be necessary to state completely both the time-action and the concentration-action relations of the system. Short of this, four characteristic pA values have been selected in the present study which together give some indication of the change of activity of an antagonist with concentration and with time. The

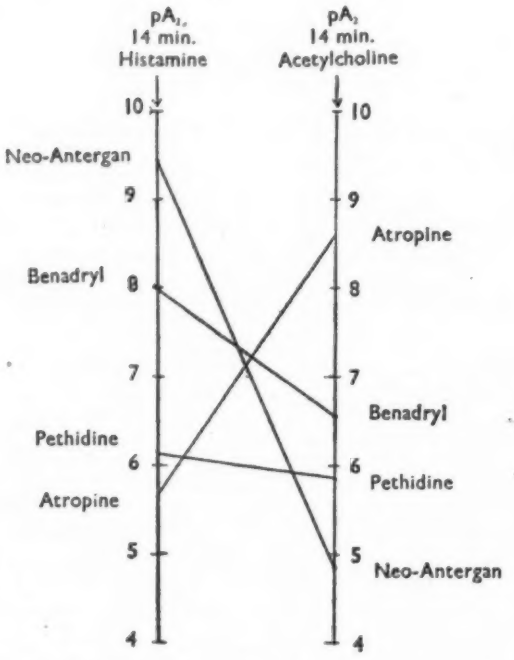

FIG. 7.—pA_2 scales of histamine and acetylcholine. At the time of measurement the antagonist had been in contact with the tissue for 14 min. The results may be taken to represent approximately equilibrium conditions.

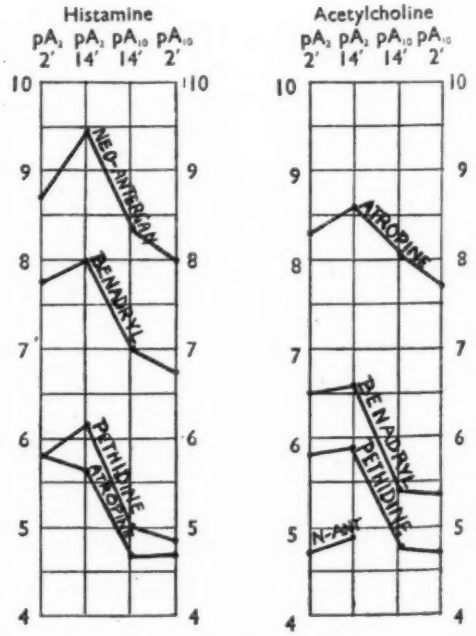

FIG. 8.—Each antagonist of histamine and acetylcholine is characterized by four pA values. Note qualitative differences between antagonists with regard to increase of action with time and pA_2–pA_{10} difference.

following four pA values have been selected for this purpose: the pA_2 values after 2 and 14 min. contact and the pA_{10} values after the same two periods of contact.

A 14 min. period of contact with the antagonist (corresponding to five consecutive contractions in the presence of an antagonistic drug) has been arbitrarily chosen as representing approximately equilibrium conditions, since at that time there is usually not much further increase of depression. It would, of course, be preferable always to establish true equilibrium conditions, but it was found that in some instances the effect of the antagonist appeared to increase indefinitely; when this happens it becomes difficult to distinguish between the effect of the antagonistic drug and that of a spontaneous deterioration of the preparation, and true equilibrium conditions cannot be established.

A convenient method of summarizing the four pA values is shown below:

	Atropine—Acetylcholine				*Pethidine—Acetylcholine*		
	pA_2	pA_{10}	pA_2–pA_{10}		pA_2	pA_{10}	pA_2–pA_{10}
2′	8.37	7.72	0.65	2′	5.79	4.69	1.10
14′	8.77	8.05	0.72	14′	5.89	4.71	1.18
14′–2′	0.40	0.33		14′–2′	0.10	0.02	

The data are taken from three experiments in which pethidine was tested against acetylcholine and three further experiments in which atropine was the antagonist, each experiment being complete in the sense that all the four pA values were determined in the course of a single assay. When the data are tabulated in this manner the row differences provide a measure of the steepness of the concentration-action curve, and the column differences indicate changes of activity with time. Pethidine and atropine differ in both these respects. The row differences are of the order of 0.7 for atropine and of 1.1 for pethidine, indicating that in order to compensate for a fivefold rise of acetylcholine the concentration of atropine has to be raised fivefold and that of pethidine thirteenfold. The difference is statistically significant. The differences in the columns show that after 14 min. contact the effect of atropine is over twice that after 2 min. contact, whilst the effect of pethidine increases hardly at all during this period.

The difference between pA_2 and pA_{10} provides a quantitative test for the hypothesis that antagonists compete with drugs for receptors according to a simple mass action relation. It can be shown that the mass action equation as developed by Gaddum (1937) for a first order reaction requires a ninefold increase of antagonist corresponding to a fivefold increase of active drug between pA_2 and pA_{10}. Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation.

The results of pA determinations are summarized in Table II. Each drug-antagonist pair is characterized by four pA values. The figures given are mean values, the total number of individual determinations and their standard deviation being indicated in the Table. Fig. 8 shows some of the differences between antagonists as revealed by the measurement of four pA values for each. Apart from differences in their general activity, antagonists also show characteristic differences in time-action and concentration-action relations. The lines joining the outer scales to the two inner scales represent increases of activity with time; these are greatest in the two most active antagonists. The lines joining the two inner scales indicate differences between pA_2 and pA_{10} (at approximately equilibrium conditions). This difference is smallest with the pair atropine-acetylcholine, which provides the only instance of straight proportionality between concentration of drug and antagonist. In all other cases a tenfold or greater increase of antagonist concentration is required to balance a fivefold increase of drug concentration.

One of the most interesting findings has been a complete lack of correlation in the behaviour of the same antagonist when tested against two different drugs. This applies both to time-action and concentration-action relations. Thus, the effect of neoantergan after the first 2 min. contact increases little further against acetylcholine, but continues to increase steeply against histamine. As for concentration-action curves, atropine has a steep concentration-action curve against acetylcholine and a relatively flat one against histamine. These results suggest that when the same antagonist antagonizes two different drugs the mechanisms involved may be quite different.

After-effects of antagonists.—The rate of recovery after the antagonist has been removed from the bath varies in much the same way as the rate of development of the effects, and here again the same antagonist may be persistent when assayed against histamine and non-persistent against acetylcholine and *vice versa.* Fig. 5 shows examples of slow (neoantergan) and quick (benadryl) recovery from antagonists on the same preparation.

A curious after-effect which occurred at times, especially with neoantergan, is shown in Fig. 9 (p. 205): the maximum depression of the effect of histamine did not take place in the presence of neoantergan, but shortly after it had been removed from the bath, as if the act of washing out the antagonist had further increased its effect.

Variability of pA Determinations

Determinations of pA_2 pethidine-histamine were made in 19 different experiments spread over a period of over one year. The results were distributed as shown in Fig. 10. The variations of pA indicate a 3.2-fold (or, omitting one result, 2.4-fold) variation in the sensitivity of the tissue. The shape of the

distribution curve suggests sampling from a non-homogeneous population. In 10 of these experiments two or more pA_2 determinations were made on the same gut and consequently variation between animals could be compared with variation in successive tests on the same animal by means of Fisher's Z test. The resulting Z value was highly significant, suggesting that there is true variation between guinea-pigs in their sensitivity to antagonists.

TABLE II

RESULTS OF pA DETERMINATIONS

The total number of individual determinations is given in parentheses and σ is the standard deviation.

Active drug	Antagonist	pA_2		pA_{10}	
		2 min.	14 min.	2 min.	14 min.
Acetylcholine	Atropine	8.27 (11) σ = 0.11	8.61 (11) σ = 0.15	7.72 (3) σ = 0.07	8.05 (3) σ = 0.13
	Benadryl	6.49 (3) σ = 0.07	6.57 (3) σ = 0.09	5.36 (2) σ = 0.06	5.4 (2) σ = 0.04
	Pethidine	5.79 (3) σ = 0.07	5.84 (4) σ = 0.14	4.7 (4) σ = 0.05	4.76 (5) σ = 0.15
	Neoantergan ..	4.71 (2) σ = 0.06	4.86 (2) σ = 0.09		
Histamine	Neoantergan ..	8.71 (4) σ = 0.15	9.46 (5) σ = 0.22	7.99 (1)	8.36 (1)
	Benadryl	7.75 (8) σ = 0.1	8.02 (9) σ = 0.28	6.74 (4) σ = 0.11	7.02 (4) σ = 0.31
	Pethidine	5.78 (19) σ = 0·14	6.13 (5) σ = 0.46	4.84 (5) σ = 0.17	5.0 (5) σ = 0.21
	Atropine	5.73 (3) σ = 0.22	5.64 (3) σ = 0.18	4.63 (3) σ = 0.24	4.6 (3) σ = 0.24

pA measurements are more variable after long periods of contact with the antagonist than after short periods. The following standard deviations summarize the variation encountered for the four different types of pA measurements:

	Standard deviation	Degrees of freedom
pA_2 2 min. contact*	0.13	31
pA_{10} 2 min. contact	0.14	15
pA_2 14 min. contact ...	0.25	34
pA_{10} 14 min. contact ...	0.22	16

*Omitting pethidine-histamine.

The standard deviation is nearly twice as great after 14 min. as after 2 min. This is somewhat surprising, since variation might be expected to become less as equilibrium conditions were approached. The increase in variability may be partly due to spontaneous changes in sensitivity of the preparation during the longer period of contact.

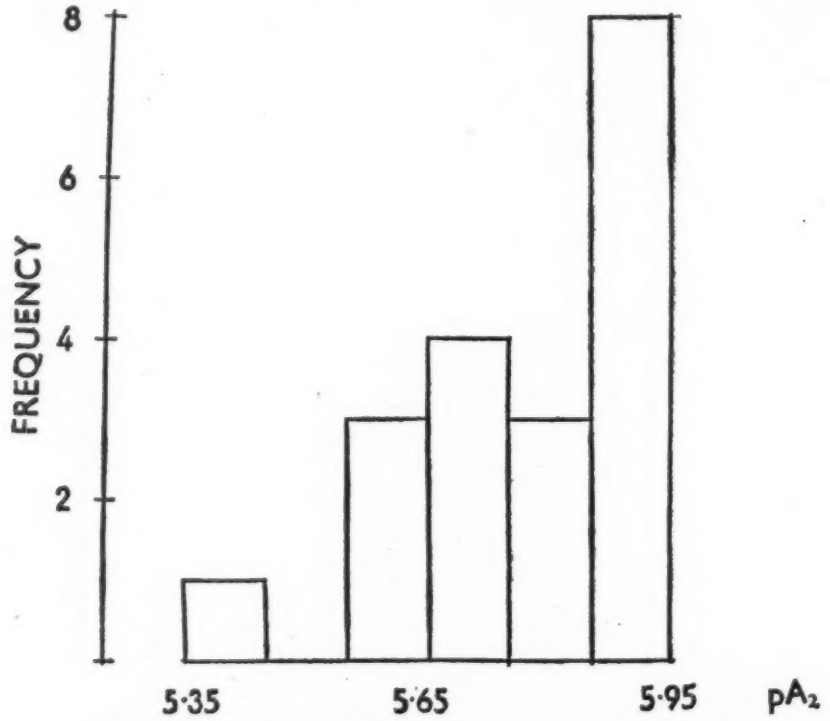

FIG. 10.—Frequency distribution of pA_2 values for pethidine-histamine obtained after 2 min. contact between drug and antagonist; 19 experiments.

THE USE OF COMPARATIVE ASSAYS FOR DETERMINING pA_2

It has been shown that animals vary in their sensitivity towards individual antagonists and that this variation is greater when the period of contact of antagonist with tissue is long than when it is short. It seemed possible that some of this variability might be eliminated in a comparative assay in which one antagonist was pitted against another antagonist. Such an experiment, involving repeated injections of each antagonist, might possibly be carried out on a single piece of gut so long as excessive depression by the antagonists was prevented by keeping the periods of contact short and by giving numerous "recovery" injections of the stimulant drug, after washing out the antagonist.

Six experiments were performed in which the action of pethidine towards histamine was compared with that of atropine on a single preparation. The experimental plan of these assays was similar to one previously used by the author (Schild, 1942) for histamine

assays. In the histamine assay only two concentrations of the standard and two of the unknown are used. These concentrations are given in a random order in successive "randomized groups" of four, and the results are eventually computed and analysed statistically on the assumption of a linear relation between log dose and effect. In the present experiments the place of four concentrations of histamine is taken by two concentrations of each antagonist administered 2 min. before the injection of a constant dose of histamine. In addition several injections of histamine alone are given following each administration of the antagonist until the sensitivity to histamine has been apparently restored. Fig. 11 shows parts of two such experiments. In both cases a "randomized group" of four doses of antagonist is represented, a complete experiment consisting of several (2–4) such "random-

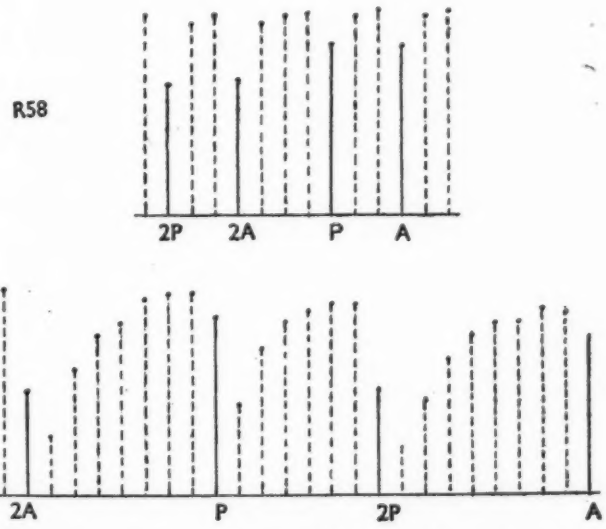

FIG. 11.—Comparisons of activity of pethidine and atropine towards histamine on a single piece of gut. Extracts from two such experiments. The solid lines represent effects of histamine in the presence of an antagonist which has previously been in contact with the tissue for 2 min., the dotted lines the effects of the same dose of histamine alone administered at 3 min. intervals until sensitivity is restored. As far as possible approximately equally depressant doses of pethidine and atropine were used, pethidine being more than twice as active as atropine. The final ratio of activity is calculated by assuming a common slope for the two antagonists and a linear relation between log concentration and effect.

ized groups." In one of these experiments the antagonists produced little after-effect; in the other a prolonged depression necessitating many "recovery" injections. In all the experiments statistical analysis showed satisfactory parallelism between atropine and pethidine slopes. The results of these comparisons, which were done at various times and on different stocks, were remarkably constant. The following figures of the logarithm of the ratio pethidine/atropine were obtained: 0.33, 0.36, 0.37, 0.34, 0.3, 0.33.

pA_2 atropine-histamine by direct and indirect method.—pA values may be determined directly without reference to another antagonist, or indirectly, by measuring in a comparative assay, such as the one outlined above, the activity

of an unknown antagonist in relation to one whose pA is already established, and computing the unknown pA by adding to the known pA the logarithm of the ratio of molar activity of the two antagonists. Thus:

$$pA_2 \text{ (atropine-histamine, 2 min.)} = pA_2 \text{ (pethidine-histamine, 2 min.)} - 0.34$$
$$= 5.78 - 0.34 = 5.44$$

This method would seem to be economical provided that the results agree with those of direct determinations ; this, however, is not entirely the case. There is a slight discrepancy between direct and indirect pA determinations which suggests that, although in comparing the activity of antagonists on a single piece of gut a 2 min. period of contact was adhered to, the gut responded in fact as if the period of contact had been longer. Indeed, the results agree better with direct pA_2 determinations done after 14 min. contact, as the following data show:

	pA_2 (2 min. contact)	Comparison on same gut (2 min. contact)	pA_2 (14 min. contact)
Pethidine-histamine	5.78		6.13
Atropine-histamine	5.73		5.64
Log ratio $\frac{\text{pethidine}}{\text{atropine}}$	0.05	0.34	0.49

These results might be explained by assuming that in comparing antagonists on a single preparation they are never completely removed in spite of repeated washings.

In comparing two antagonists on the same gut the result may be vitiated by certain systematic errors unless care is taken that the effect of a previous dose has subsided at the time the next dose is added, especially when the two antagonists have different modes of action. The following example illustrates the point.

Antagonist (a) is to be compared with antagonist (b); their effects are additive but (a) has a persistent action and (b) a readily reversible action. When (a) is added to the bath some of its effect persists after it has been removed. If a depression is produced by (a) and matched at the next injection by a depression produced by (b) the real effect of the latter, acting as it does on a depressed preparation, is less than it appears. At the next injection the same concentration of (a) reproduces its old action, the effect of (b) having worn off.

Comparison of antagonists on separate pieces of the same gut.—In these experiments direct pA determinations on two different antagonists were done simultaneously using a separate piece of gut for each antagonist and each concentration, all the segments being taken from the same guinea-pig. Variability between guinea-pigs was thus eliminated.

An experiment of this kind comparing the action of neoantergan and benadryl towards histamine is illustrated in Fig. 5. The results of this and two further similar experiments are shown in Table III.

The following points may be noted:

(i) The difference between pA_2 values at 2 min. contact is remarkably constant, more constant than the absolute values. This confirms previous conclusions that the gut varies simultaneously in sensitivity towards different antagonists.

(ii) Increase of activity with time (pA_2 14 min.–pA_2 2 min.) varies, but it is in each case considerably greater with neoantergan than with benadryl.

(iii) At 14 min. pA_2 values and their differences vary more than at 2 min. This confirms previous conclusions.

In conclusion it may be said that comparative measurements definitely eliminate a certain amount of variation. Comparisons on a single piece of gut

TABLE III

pA_2 NEOANTERGAN-HISTAMINE AND BENADRYL-HISTAMINE DETERMINED IN THE SAME EXPERIMENT USING A SEPARATE PIECE OF ILEUM FOR EACH CONCENTRATION OF ANTAGONIST

		pA_2 values		pA_2 neoantergan —pA_2 benadryl
		neoantergan	benadryl	
R.205	2′	8.91	7.94	0.97
	14′	9.76	8.35	1.41
	14′–2′	0.85	0.41	
R.206	2′	8.61	7.66	0.95
	14′	9.60	7.78	1.82
	14′–2′	0.99	0.12	
R.207	2′	8.65	7.75	0.90
	14′	9.36	8.26	1.10
	14′–2′	0.71	0.51	

are efficient in the sense of allowing many determinations to be made in a relatively short time, but they may be affected by systematic errors owing to persistence of antagonistic effects. Comparisons on separate pieces of gut are laborious but free from objections and give a more complete picture, since the time factor may be taken into account.

Discussion

Clark and Raventos (1937) proposed to use as a measure of activity of an antagonist the concentration which would neutralize the effect of a tenfold increase of active drug. One of the chief merits of this measure is that, being a null measure, and involving no change in response, it is independent of the method of experimentation used, and yields results on different preparations which are directly comparable.

pA is based on the same idea as the measure adopted by Clark and Raventos, but it is expressed in a rather more convenient form. It may be used to define the activity and specificity of an antagonist, its time-action relations and the trend of its concentration-action curve. pA values are additive; for instance, in order to express the total activity of a compound against both histamine and acetylcholine, the respective pA values may be added together.

The activity of an antagonistic drug may be expressed in one of two ways, by reference to another antagonist or by some measurement not involving a comparison with another drug. Since the activity of a drug cannot as a rule be expressed accurately in terms of another drug which is qualitatively different, the comparative method gives an incomplete and often misleading picture of the activity of an antagonist. Moreover, unless a definite common standard has been agreed upon, results from different laboratories are difficult to correlate. Comparisons may nevertheless be useful in practice owing to their greater economy of time and effort, especially when a series of related compounds is being investigated within the same laboratory. This question has been discussed in detail above.

The real test of the usefulness of a measure of drug antagonism is whether the results can be reproduced in another laboratory. Variations occurring within the same laboratory can be overcome by repeating the experiment and by random selection of experimental animals, but systematic variations between different laboratories present a more difficult problem. At present it is not known whether pA will be affected by such systematic variations or whether results in different laboratories will fall within the range of variations obtained with a highly mixed stock of animals in an individual laboratory. If important variations in sensitivity between laboratories should occur, one way of eliminating them would be to use in this type of work a homogeneous strain of guinea-pigs, available to all the laboratories concerned. Failing this, it might become necessary to fall back on the method of establishing differences of pA between antagonists rather than absolute pA values in the hope that the former would be less subject to laboratory variation than the latter. The use of an agreed measure of drug antagonism should, at least, make it easier to detect the occurrence and extent of such variations.

Summary

1. Clark and Raventos (1937) suggested a method of estimating antagonistic power in terms of "the concentration of antagonist which altered by a selected proportion (e.g., tenfold) the concentration of an active drug needed to produce a selected effect." The negative logarithm of this (molar) concentration has been termed pA_x, where x is the proportion selected. On the guinea-pig's ileum the value of pA appears to be independent of the degree of contraction produced by the active drug. Methods are described for determining the value of pA.

2. pA is a statistical constant. To obtain a representative value of pA for a given tissue, antagonist, and active drug, the mean of a random sample of determinations on different individual animals must be determined. pA values were found to vary by 0.4 to 0.5 of a unit when the period of contact of the muscle with the antagonist was short; at long periods of contact variability was increased. Variability may be reduced by comparing the activity of one

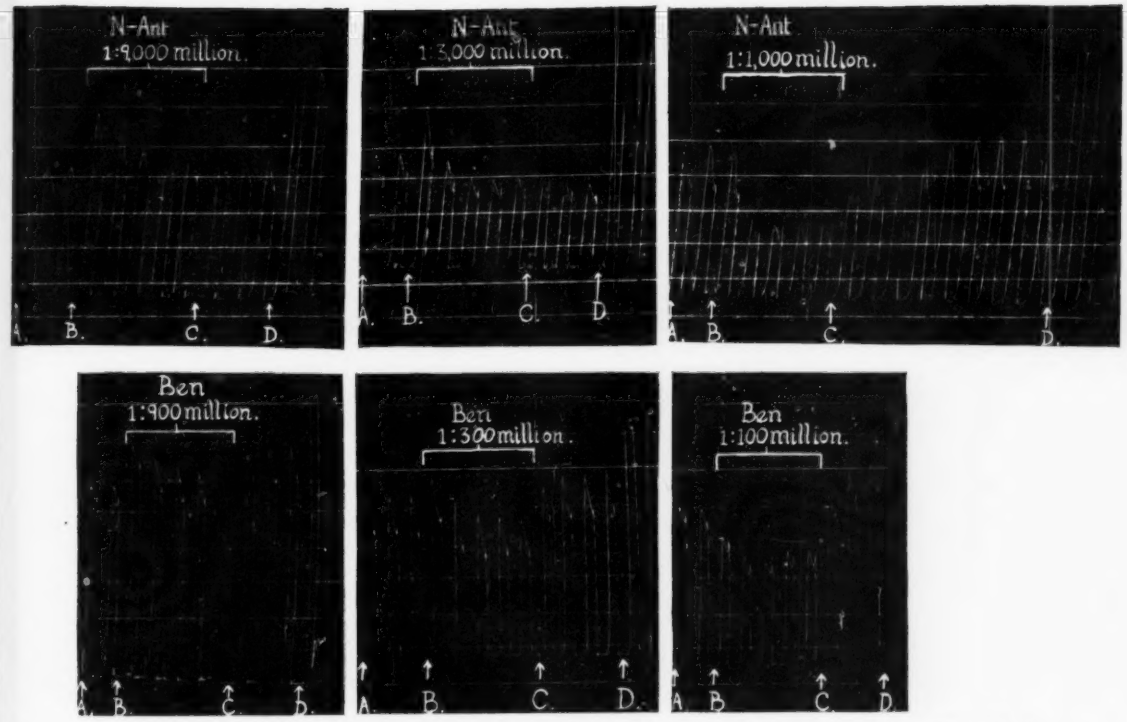

FIG. 5.—Neoantergan-histamine and benadryl-histamine. Complete pA_2 assay. The whole assay of the two antagonists was done on the same animal ; a separate piece of ileum was used for each concentration of antagonist. Before the addition of the antagonist the preparations were stabilized by a series of preliminary histamine injections. A–B: 0.5 μg. histamine, Tyrode. B–C: 1.0 μg. histamine, antagonistic solution. C–D: 1.0 μg. histamine, Tyrode. D: maximum effects. Bath volume = 18 c.c.

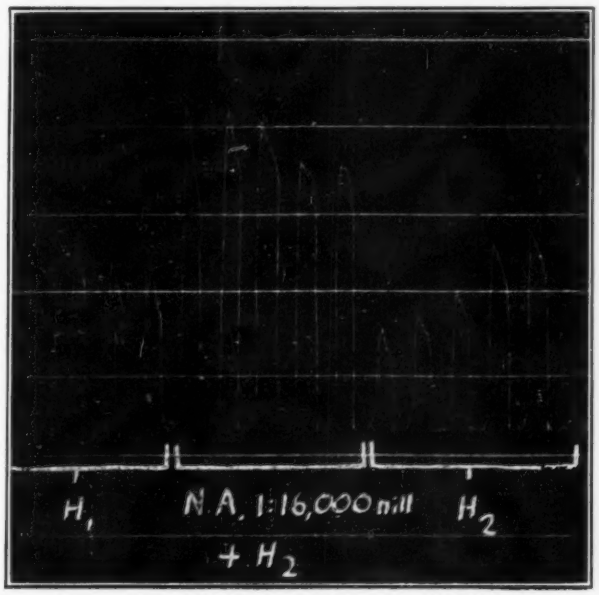

FIG. 9.—Neoantergan-histamine. After-effect of antagonist. Immediately after removal of the antagonistic drug from the bath there is a further increase of depression. H_2 = double dose of histamine.

antagonist with that of another. Methods are described for making such comparisons in an efficient way and possible objections to a comparative method are discussed.

3. The activity of neoantergan, benadryl, pethidine, and atropine in antagonizing histamine and acetylcholine has been determined in terms of pA on the guinea-pig's ileum. All these antagonists act against both histamine and acetylcholine, though at widely differing concentrations and in a qualitatively different way, as shown, for instance, by the time taken for equilibrium conditions to be reached. By characterizing each drug-antagonist pair by four pA values a more complete picture can be obtained of activity as influenced by duration of action and by concentration.

I am indebted to Dr. F. Bergel, of Roche Products, Ltd., for supplying Pethidine ; to Dr. J. S. White, of Parke Davis & Co., for Benadryl ; and to Dr. R. Wien, of May & Baker, for Neoantergan. The special armatures for converting P.O. relays into fluid switches were designed and made by Mr. B. F. Ballhatchet. The drawings are by Mr. A. Boura.

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SOME ACTIONS OF β-HYDROXY-αβ-DIPHENYLETHYLAMINE

BY

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Morphine being a pharmacologically active derivative of phenanthrene Dodds, Lawson, and Williams (1944a) sought a morphine analogue on the same lines as their discovery of stilboestrol—an intensely active analogue of another phenanthrene derivative, oestradiol. They found that diphenylethylamine and seventeen related compounds possessed in differing degrees some of the properties of morphine ; these were depression of righting reflex in rats, elevation of blood sugar in rabbits, and hyperexcitability, pupil dilatation, and vomiting in cats. Some compounds of this series also possessed some analgesic properties when tested clinically. Of these the most promising was diphenylethanolamine or β-hydroxy-αβ-diphenylethylamine, which may be considered to have the same structural relation to morphine as stilboestrol has to oestradiol. Later (Dodds *et al.*, 1944b) it was shown to be effective only in cases where the pain was associated with pressure on nerves. The possible therapeutic uses of this substance, called M4 in their series, justified an investigation of its general pharmacological actions.

Tiffeneau, Levy, and Boyer (1928) found that diphenylethanolamine caused weakening of the beat and slowing of the isolated snail and frog heart, and also of the exposed heart of chloralosed dogs. The substance also caused relaxation of isolated intestine, transient fall of arterial pressure in chloralosed dogs, but gave a vasoconstriction of the perfused isolated frog leg. Hasama (1930) showed that the fall of blood pressure induced by the substance in urethanized rabbits was still obtained after vagotomy or atropine. The vessels of the isolated rabbit ear were dilated by diphenylethanolamine, even in the presence of atropine ; since the dilator action could antagonize barium chloride vasoconstriction Hasama suggested that the activities of the substance were the result of a direct toxic action on smooth muscle. Tainter (1933) and Dodds,

Lawson, and Williams (1944a) also noted the depressor action and the latter authors commented on the dilatation of the pupil and general hyperexcitability of unanaesthetized cats following intramuscular injection of the substance.

In this paper is presented confirmation and extension of the previous work with an analysis of the site of action of the substance. The actions of β-hydroxy-$\alpha\beta$-diphenylethylamine hydrochloride, which will be referred to as M4, were tested on the following mammalian tissues: (*a*) small intestine, (*b*) cardiovascular system, (*c*) the pupil of the eye.

Methods

For studies on isolated intestines and heart, rabbits were killed by stunning. Pieces of small intestine were suspended in a bath of oxygenated Tyrode solution (formula in Bayliss, 1924) at 37–38° C. and *p*H 7.4, and the contractions of the longitudinal muscles recorded. The coronary arteries of the heart were perfused with oxygenated Ringer-Locke solution (formula in Bain, 1938) at 37–38° C. and *p*H 7.4, through a cannula in the aorta; movements of the right ventricle were recorded, the heart being steadied by pinning the apex. For perfusion of the hind quarters, rabbits and cats were used; the former were killed by stunning, the latter had been anaesthetized with chloralose (60–80 mg./kg. intravenously) for other experiments. A cannula was inserted in the lower aorta and the legs were perfused with Ringer-Locke solution at 38° C. and *p*H 7.4 by a Dale-Schuster perfusion pump, the inflow pressure being recorded with a mercury manometer.

Blood pressure changes were recorded in cats anaesthetized with chloralose (60 mg./kg. intravenously in preliminary ether anaesthesia) or with nembutal (0.5 c.c. nembutal solution (Abbott)/kg. intraperitoneally). Carotid arterial pressure was recorded by a mercury manometer. To assess the action of M4 on the vessels of different tissues the volumes of a hind paw, of the opposite skinned hind leg, and of a 2-in. length of small intestine were recorded optically on photosensitive paper (Downman, Goggio, McSwiney, and Young, 1943); in these experiments the arterial pressure was also recorded optically on the same paper. The organs were enclosed in plethysmographs, the leg plethysmograph enclosing the skinned leg from upper thigh to ankle, but the skin of the same paw and its venous drainage were left intact outside the plethysmograph.

For experiments on the pupils of cats, slit-like pupils were produced in two ways. First, the animals were anaesthetized with chloralose (80 mg./kg. intravenously) given in preliminary ether anaesthesia (McDowall, 1925; Bain, Irving, and McSwiney, 1935). Secondly, under ether anaesthesia cats were decerebrated through a trephine opening in the cranium, the plane of section curving downwards and forwards from the upper edge of the inferior colliculi to the sphenoid eminence, leaving the hypothalamus and adjoining structures intact. The carotid arteries were temporarily occluded by clips and the vertebral arteries by digital pressure, but they were released as soon as possible. As the animal excreted its ether the pupils closed down to slits. If restoration of blood flow to the brain stem was delayed too long the pupils might not constrict. The occulomotor nerve could be exposed by tearing the dural covering over it on its way to the orbit.

Stock solutions of M4 were made by dissolving the hydrochloride in distilled water, without heat, to give 2 or 5 per cent (w/v) solutions. Dilutions were made from the stock solution into the appropriate physiological salt solution. It should be noted that solutions of M4 are acid, and simple neutralization throws the base out of solution. All doses are in terms of the hydrochloride. Injections into the cat were made into the superficial vein of the right foreleg.

RESULTS

(a) Small intestine

Isolated small intestine of rabbit was relaxed by M4 and the rhythmic movements diminished in amplitude. The lowest concentration found effective was 1 in 20,000. The loss of tone was rapid at first, but was followed by prompt recovery of tone and activity on washing (Fig. 1). M4 was active on the atropinized intestine and antagonized the spasm produced by acetylcholine, eserine, prostigmine, and barium chloride. Whereas M4 relaxed the gut, similar concentrations of morphine sulphate produced a slow increase of tonus.

FIG. 1.—Action of hydroxydiphenylethylamine upon longitudinal movements of rabbit duodenum suspended in Tyrode solution. M4 = addition of 2 per cent solution to give a final solution of 1:5,000. W = wash. Time signal = 30 sec.

Aqueous solutions of M4 being acid—e.g., *p*H 5.3 for 2 per cent (w/v) solution—it might be argued that the spasmolytic action represents no more than the action of an acid solution. Indeed there was a noticeable fall of *p*H, shown by adding phenol red to the Tyrode solution, after addition of an active quantity of M4. Advantage was taken of the buffering power of plasma. The *p*H of heparinized rabbit plasma was 7.40. After the addition of 1 volume of 2 per cent (w/v) aqueous solution of M4 to 4 volumes of plasma the *p*H of the mixture, measured with a glass electrode *p*H meter, was the same as that of the original plasma. The buffered M4 produced the same changes of intestinal activity as unbuffered M4, when each was added to the Tyrode bathing the intestine to the same final concentration of M4.

(b) Cardiovascular system

Isolated rabbit heart.—0.1 to 0.2 c.c. of 2 per cent (w/v) solution of M4 injected quickly into the perfusion cannula produced a sharp decrease of amplitude, with not more than 15 per cent slowing of the beat. The heart recovered steadily in the next 5 minutes. Similar results were recorded when the same doses of M4 were diluted with 0.8 c.c. rabbit plasma before injection, whereas plasma alone usually produced a slight increase in the amplitude of beat.

Faradizing a vagus nerve supplying the isolated heart caused slowing and weakening of the beat. At the height of the M4 action vagal stimulation produced no or but slight slowing of the beat. It was noticed also that the amplitude of the beat might be a little increased during the stimulation

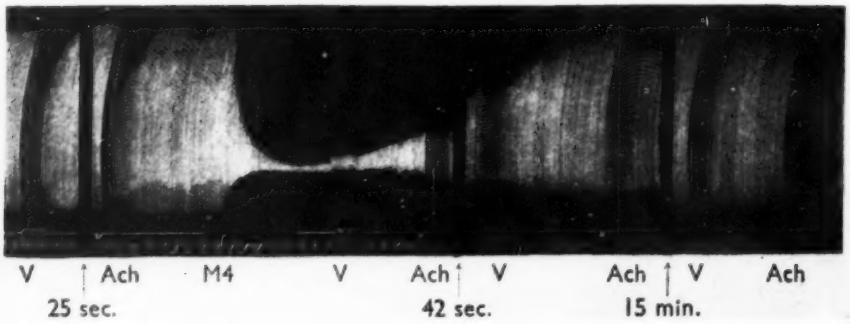

FIG. 2.—Action of hydroxy-diphenylethylamine on the isolated perfused rabbit heart. Tracing shows movements of right ventricle. M4 = injection of 2 mg. hydroxy-diphenylethylamine HCl into aortic perfusion cannula, buffered by mixing 1 vol. 2 per cent solution of M4 with 4 vol. rabbit plasma. Ach = injection of 2 μg. acetylcholine in 0.2 c.c. Ringer-Locke solution. V = faradic stimulation of vagus nerve on the oesophagus. Time tracing = 5 sec.

(Fig. 2). The vagal inhibitory action returned as the heart itself recovered from the influence of the M4, but the full return of vagal response was not seen until some 5 minutes after the amplitude of the heart beat had fully recovered. The response to acetylcholine—e.g., 1 μg. in 0.1 c.c. Ringer Locke—was also reduced, and vagal and acetylcholine action was recovered at about the same speed. M4 action on the heart was not influenced by prior atropinization. Following a depressant dose of M4 the action of adrenaline was reduced but not abolished.

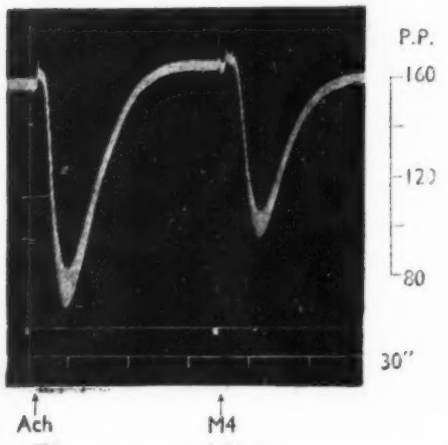

FIG. 3.—Vasodilator action of hydroxy-diphenylethylamine and of acetylcholine compared in cat hindquarters perfused Ringer-Locke solution, *p*H 7.5, containing 1 : 500,000 adrenaline. Ach = 3 μg. acetylcholine. M4 = 2 mg. hydroxy-diphenylethylamine HCl. Time signal = 30 sec. Perfusion pressure (P.P.) in mm. Hg.

Perfused hind limb.—In order to demonstrate the action of M4 or any vasodilator drug the tone of the vessels was raised by adding adrenaline to the perfusion fluid in a concentration of 2×10^{-6} to 3×10^{-7}. M4 now produced a transient dilatation of the limb vessels, shown by a fall of the perfusion pressure head (Fig. 3). This dilator response, which was mimicked by 0.05 to 0.1 c.c. $N/10$ hydrochloric acid, cannot be attributed solely to the injection of an acid solution. It was obtained when the M4 was buffered adequately with plasma, as described above, although plasma injected alone produced a small rise of perfusion pressure.

The dilator action of M4 was seen when the limb vessels were constricted not only by adrenaline but also by posterior pituitary extract or barium chloride.

Vascular responses in anaesthetized cats.—Intravenous injection of M4 dissolved in 0.9 per cent sodium chloride solution produced a temporary fall of general arterial pressure (Fig. 4). With small doses arterial pressure recovered

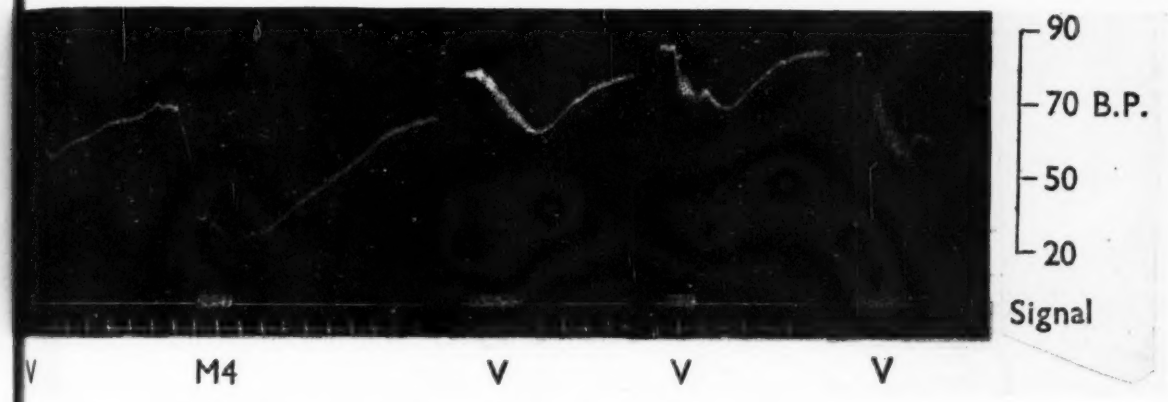

FIG. 4.—Depressor action of hydroxy-diphenylethylamine, 20 mg. i.v., upon carotid arterial pressure of chloralosed cat. (Signal for injection is 10 sec. late.) V = faradic stimulation of peripheral end of cut right vagus nerve in neck. Time tracing = 10 sec. B.P. calibration in mm. Hg.

quickly and was usually followed by a small but prolonged hypertension. In chloralosed cats 10 mg. M4/kg. produced about a 40-mm. Hg fall of blood pressure with recovery in two minutes, while 50 mg./kg. led to a 75-mm. Hg fall of pressure with cardiac irregularity. Doses over 50 mg./kg. caused cessation of breathing for periods up to 25 minutes.

Accompanying the fall of arterial pressure there was a rapid decrease of volume of the paw, skinned limb, and intestine. At the same time the pulsations in these organs decreased in amplitude. As the arterial pressure recovered the organ volume and the amplitude of the pulsations returned. Similar changes were seen after bilateral vagotomy in the neck and inactivation of both carotid sinuses by tying the arterial trunks close to the sinus where they entered and left the structure. The shrinkage of the peripheral organs started at the same time in each of them and did not commence until the central arterial pressure had already fallen a little (Fig. 5).

The loss of vagus action in the presence of M4 could be shown in the intact animal by comparing the depressor response to faradizing the peripheral end of the cut right vagus nerve in the neck before and after injection of a dose of M4 which produced a prolonged action. The fall of pressure became smaller and the vagal inhibition of heart rate was much reduced; as in the isolated

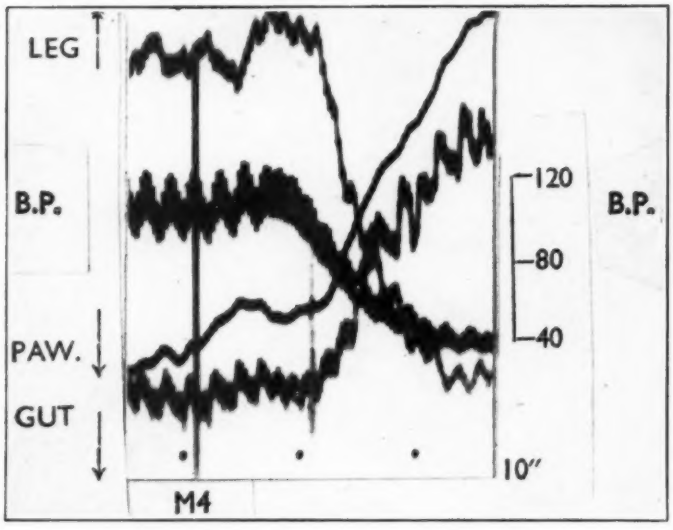

FIG. 5.—Optical record of carotid arterial pressure, with volumes of left hind paw, skinned right hind leg, and segment of jejunum. Cat, nembutal i.p. At signal 30 mg. hydroxy-diphenylethylamine i.v. Time marking = 10 sec. B.P. calibration in mm. Hg. Arrows show direction of vasodilatation.

heart, as blood pressure recovered so the vagus responses recovered, but the latter was not complete until some 1 to 5 minutes after the end of the M4 depressor response (Fig. 4).

(c) Action on the pupil

In the chloralosed or decerebrated cat with slit-like pupils intravenous injection of M4, in doses of 12 mg./kg. or more, produced a rapid wide dilatation of the pupil with slow recovery. The whole effect lasted 10 to 30 minutes, and was not altered by cutting both cervical sympathetic chains in the neck. In cats with one oculomotor nerve severed it was possible to follow the dilator action of M4 upon the contralateral normal eye, and at the same time to test the ability of the oculomotor nerve to constrict the pupil by faradizing the peripheral end of the cut nerve. With the nerve severed the pupil was widely dilated, but constricted to a slit when the nerve was stimulated. At the height of the M4 action, as judged by the dilatation produced in the normally innervated eye, the constrictor effect of oculomotor stimulation was quite absent. As the pupil dilator action of M4 receded, so the ability of oculomotor nerve impulses to produce pupil constriction returned. Even when oculomotor action on the pupil was paralysed, stimulating the nerve still produced the usual rotation of the eyeball and enophthalmos. Stimulating the peripheral cut end of the cervical sympathetic chain in the neck produced a further slight dilatation

of the pupil when the latter was apparently fully dilated by M4, as well as movement of the nictitating membrane.

M4 could antagonize the action of eserine. This was shown by cutting the oculomotor nerve on one side and then constricting the paralysed pupil by instilling into the conjunctival sac 0.65 mg. eserine sulphate (1 Burroughs Wellcome "Tabloid"). Intravenous injection of a dose of M4, which produced full dilatation of the normal pupil, produced one-third dilatation of the eserinized pupil.

Stereoisomers of hydroxy-diphenylethylamine

There being two assymmetric carbon atoms in the molecule two optically inactive stereoisomers are possible, the normal form (M4 itself) and an *iso* form. Each of these can be resolved into two optically active enantiomorphs. A comparison of the activities of these various isomers was attempted, using rabbit jejunum as the test object. More regular responses were produced if the gut was brought into high tone by suspending it in Tyrode solution to which eserine sulphate was added in a concentration of 1 : 4 million. The isomers were added to the gut bath as 2 per cent (w/v) solutions in water in an amount producing about half the maximum relaxation of the gut. The gut was exposed to each isomer for 5 minutes, then washed twice with Tyrode solution in the next 10 minutes. Even with this long exposure some pieces of gut did not reach their final length as the relaxation was rapid for the first two or three minutes and then proceeded very slowly.

Comparing the relaxation produced by similar concentrations of the isomers, 1 : 15,000, the activities of the isomers could be listed as *l-iso*>*d*-normal>*dl-iso* >*dl*-normal>*d-iso*. The *l*-normal form was not available. Equal relaxation was produced by 1 : 20,000 of the *l-iso* and 1 : 12,000 of the *d-iso*. It is difficult to assess the value of these results. Although there is a consistent difference between the *l-iso* and *d-iso* form, the activities of the first four isomers listed are very similar. Comparison of the vasodepressor action in the chloralosed or spinal cat was less successful. The responses to similar doses of the isomers were not always consistent, and might change during the experiment, but the results did not suggest any such difference of activity as shown by the gut.

Discussion

Although hydroxy-diphenylethylamine (M4) apparently resembles morphine in having analgesic action, Dodds, Lawson, and Williams (1944) showed that these two substances probably act in different ways. Similarly, the previous results show that the general activities are also different. M4 is in general a depressor of smooth muscle action. Also it has an action which may for the moment be called "atropine-like."

It has been shown that the drug relaxes intestinal muscle and reduces the amplitude of the rhythmic contractions. This action is reversible and can be exerted against substances, such as barium chloride, which raise the tonus of the muscle. A similar spasmolytic action is produced in the blood vessels. These actions might all be explained by a general toxic action which directly reduces the power of the muscle to contract. Such a depressant action is seen in the heart, where M4 reduces considerably the amplitude but not the frequency of the beat. That this effect is not due to a parasympathomimetic action of the drug is shown by its occurrence in the atropinized heart. These findings agree with the reports of previous workers.

In view of the depressor action of M4 it could be argued that the fall of arterial pressure is a consequence of a peripheral vasodilatation, especially as M4 does have such an action in the isolated perfused limb. The simultaneous recording of paw, skinned limb, and intestine volumes shows however that in the whole animal the fall of arterial pressure is accompanied by a shrinkage of these organs. Since M4 is an active vasodilator in the isolated perfused limb, with maintained inflow, it seems that in the whole animal there is produced a passive vasoconstriction consequent upon the fall of arterial pressure. This is confirmed by the fall of pressure starting demonstrably earlier than the volume changes of skin, skeletal muscle, or intestine. The initial rapid fall of blood pressure on injecting M4 seems, therefore, to be due to a reduction of heart output because of the toxic action of the drug on the heart muscle. This toxic action is well shown on the isolated heart preparation.

The dilator action on the pupil in the unanaesthetized cat might be due to some central action in the brain stem or to a peripheral paralysis of the iris. That the neuromuscular mechanism of the iris is influenced directly is shown by the ineffectiveness of the oculomotor nerve stimulation in the presence of a dilator concentration of M4. It is probable, therefore, that a peripheral action would explain the pupil dilatation observed by Dodds and his colleagues. It is to be noted in the paper by Dodds, Lawson, and Williams that a dose of M4 which produced general hyperexcitability (20 mg./kg. intramuscularly) was less than the pupil-dilating dose (50 mg./kg.). A general toxic action on the whole neuromuscular mechanism does not seem to be an adequate explanation of the oculomotor paralysis. The dilator action of the sympathetic fibres was still present, arguing against an inability of the muscle fibres of the iris to react to nerve impulses; further, conduction in the oculomotor nerve was not impaired because it could still carry nerve impulses to the extraocular muscles, producing movement of the eyeball. The action of M4 on the pupil seems to be of an atropine-like nature, blocking conduction at the parasympathetic terminals. This action is, however, of short duration and is produced only in concentrations which have direct toxic actions on other smooth muscle.

The ability of M4 to block the action of parasympathetic nerve impulses

is seen also in its effect upon the vagal control of heart rate. This action has been observed only after doses of M4 sufficient to produce severe weakening of the beat, but does persist for a few minutes after the visible signs of this depression have worn off. It will be recalled that certain barbiturates produce a similar transient blocking of parasympathetic impulses and there is some evidence that the site of the block may be either in the ganglion or at the neuromuscular junction. Thus Koppanyi, Linegar, and Dille (1935) showed that some barbiturates, other than thiobarbiturates, produced a transient loss of vagal action and the evidence suggests that the site of action of the drug is mainly at the vagus ganglion; Stravraky (1931) showed that amytal paralyses the submaxillary glands to chorda tympani stimulation, and since the acetylcholine response is also paralysed it appears that the drug acts on the nerve terminals or the secretory cells. Garry (1930) also has shown that amytal temporarily abolishes the action of the vagus nerve on the heart in cats and rabbits, the effect passing off much more rapidly in the rabbits; the action of acetylcholine on the frog heart was unchanged, but vagal activity was not tested at the same time in this animal. In the heart both vagal and acetylcholine activities are paralysed by M4 to the same degree, but whether this should be compared with the action of atropine is debatable, since M4 is so toxic to the heart muscle. It is possible that the effect represents no more than a general toxic action which takes a little more time to recede from the more fragile place where acetylcholine acts. The observations on the pupil, however, suggest that toxic and "atropine-like" action may be separate entities. It is curious that although M4 reduces the force of the heart beat very greatly it has relatively little action on the rate of firing of the pacemaker.

Although many of the actions of M4 may be due to non-specific depression of the tissues, there seems to be stimulation of some parts of the central nervous system. General bodily activity is increased (Dodds *et alia*, 1944), an action shown by diphenylethylamine (Tainter, Ludvena, Lackey, and Neuru, 1942); some related compounds will even cause convulsions in cats. Some of the diphenylethylamine compounds also cause a rise of blood sugar (Dodds *et alia*, 1944), but the ability to cause hyperexcitability and raise the blood sugar are dissociated in M4, this substance producing hyperexcitability without hyperglycaemia. Clearly it would be of interest to know more of the cause of the hyperglycaemia provoked by other diphenylethylamine derivatives.

Summary

A morphine analogue, β-hydroxy-αβ-diphenylethylamine, has the following actions:

1. It relaxes isolated intestine, even in the presence of acetylcholine, eserine, prostigmine, or barium chloride.

2. The contraction of the isolated heart is reduced in amplitude, this action being unaffected by previous atropinization. Loss of response to vagal impulses and to acetylcholine is also produced.

3. It causes a fall of arterial pressure, with peripheral vasoconstriction. The latter is a passive effect. In the isolated perfused limb active dilatation is produced.

4. It produces pupil dilatation in chloralosed and decerebrated cats by an action on the oculomotor nerve terminals in the iris.

5. These results might be explained by a direct toxic action of the drug on the active tissue.

This investigation was undertaken after a suggestion by Prof. E. C. Dodds, M.V.O., F.R.S., whom I have to thank for original supplies of M4 and its isomers. The M4 was made by Boots Pure Drug Co., Ltd., to whom thanks are due. During the work much valuable assistance was given by Miss J. G. Emmett, of Boots Pure Drug Co., Ltd., working in this laboratory.

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THE THERAPEUTIC ACTION OF SOME KNOWN AMOEBICIDES IN RATS

BY

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Three main types of drug are used in the treatment of human amoebiasis: firstly, drugs of the emetine type, e.g. emetine hydrochloride, emetine bismuth iodide, and auremetine; secondly, arsenical drugs, e.g. carbarsone and stovarsol; and thirdly, halogenated hydroxyquinolines, e.g. chiniofon, vioform, and diodoquin. Opinions differ concerning the respective merits of these three types; indeed the general opinion seems to be that no one type is entirely satisfactory in itself, and that the best clinical results are obtained by the judicious use of all three. As we now have a technique for evaluating the anti-amoebic properties of drugs, using experimentally infected rats (Jones, 1946), it was considered of interest to make a close comparison of the most commonly used of the above types. Such a comparison would also serve to supply standards against which any newly discovered anti-amoebic drug could be compared.

The drugs selected for the comparison were emetine hydrochloride, chiniofon, stovarsol, carbarsone, and diodoquin. They were compared when given as a single dose, and also when given according to a multiple-dose schedule.

Experimental Work

Several experiments were carried out, each involving the use of 144 recently weaned rats weighing approximately 20–33 g. In each experiment the rats were separated into six groups of matched weights. They were then injected intracaecally, after laparotomy, with 0.25 c.c. of a suspension of *Entamoeba histolytica* in 5 per cent gastric mucin. The amoebae were cultivated in the enriched serum-buffered saline medium described previously (Jones, 1946).

Two different dosage schedules were employed: in the first a single dose was given 24 hours after the operation, and in the second doses were given 24, 30, 48, 54, and 72 hours after the operation. All doses were given orally, by means of a metal catheter. Emetine and chiniofon were given as solutions in water, and stovarsol, carbarsone, and diodoquin as finely dispersed suspensions made by ball-milling for several hours with a suitable dispersing agent (1 per cent Dispersol O.G., I.C.I.).

In order to assess the therapeutic effect of the test drug the rats were killed six days after the operation. After this time the infection in the control group is maximal, and any therapeutic effect in treated groups is therefore most readily detectable. Careful post-mortem examinations were made of each rat in the experiment, and according to the degree of infection found a score was allocated. The six standard degrees of infection are shown in Fig. 1.

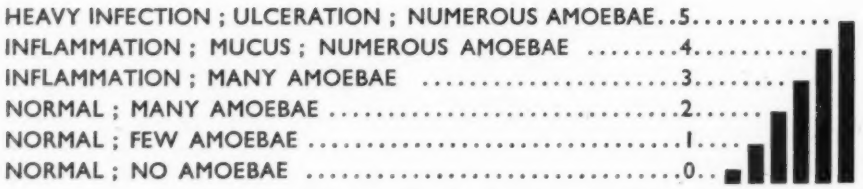
HEAVY INFECTION ; ULCERATION ; NUMEROUS AMOEBAE..5.....
INFLAMMATION ; MUCUS ; NUMEROUS AMOEBAE .....4.....
INFLAMMATION ; MANY AMOEBAE .....3.....
NORMAL ; MANY AMOEBAE .....2.....
NORMAL ; FEW AMOEBAE .....1....
NORMAL ; NO AMOEBAE .....0..

FIG. 1.—The degree of amoebic infection found in the caecum of experimentally infected rats.

An average degree of infection (ADI) was calculated for each group by finding the average of the individual scores.

The statistical significance of a treatment effect may be assessed according to the following formula:

$$z = \frac{x - y}{\sqrt{\frac{\sigma^2 x}{m} + \frac{\sigma^2 y}{n}}}$$

where x and y are the ADIs for the control and treated groups respectively.

σx and σy are the standard deviations for x and y respectively, and m and n are the numbers of rats in the control and treated group respectively.

The values of σx and σy used in the above formula were read from a curve derived from the results of a large number of control and treated groups (Jones, 1946).

In this series of experiments most of the drugs, when given at the highest dose, showed a therapeutic effect of high statistical significance ($P<0.01$). This value ($P<0.01$) was therefore taken as the standard representing definite positive therapeutic effect (+). Therapeutic effects of significance $P=0.05$ to $P=0.01$ were regarded as indicating slight though definite therapeutic effect (±); whilst effects of $P>0.05$ were regarded as indicating insignificant therapeutic effect (−).

The results of the comparisons are recorded in detail in Figs. 2 and 3, and summarized in the Table.

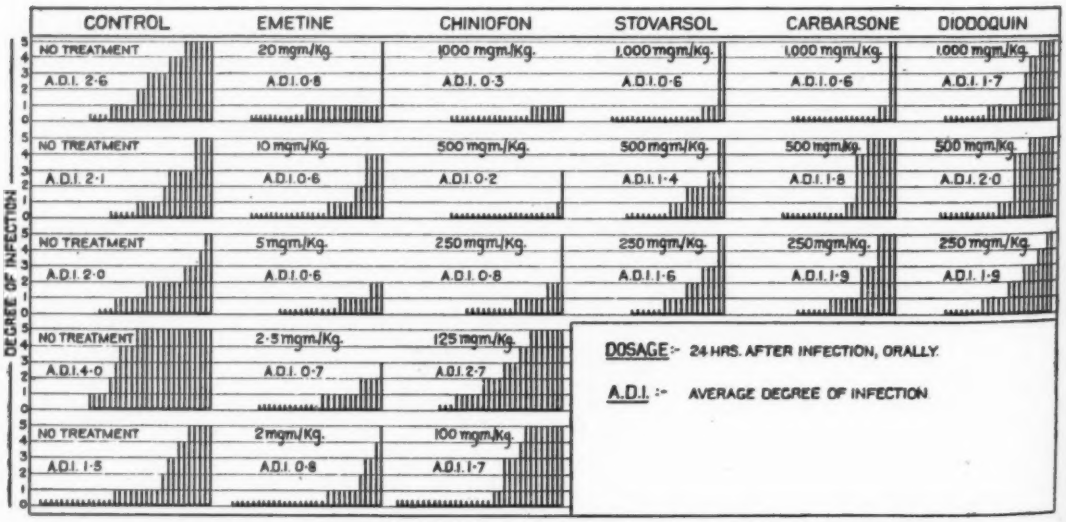

FIG. 2.—The effect of drugs on experimental amoebiasis in rats. Single-dose therapy.

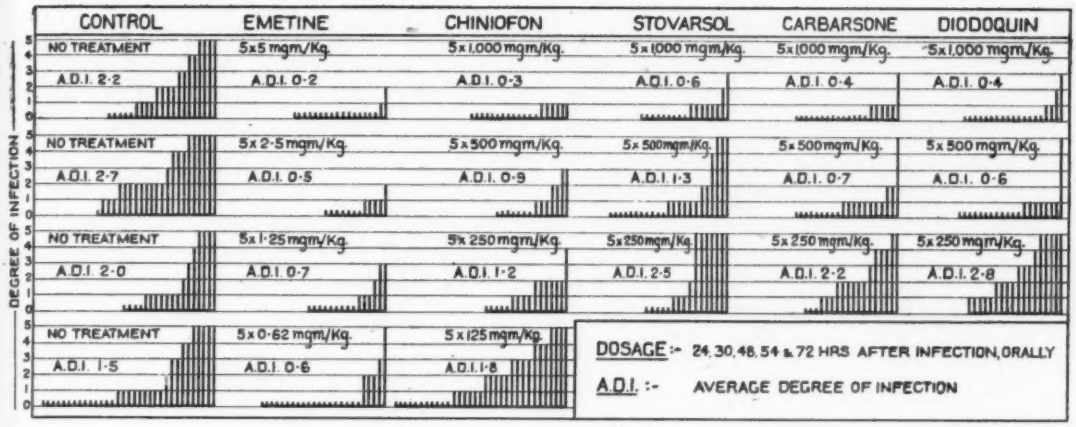

FIG. 3.—The effect of drugs on experimental amoebiasis in rats. Multiple-dose therapy.

TABLE

THE EFFECT OF DRUGS ON EXPERIMENTAL AMOEBIASIS IN RATS

	Dose, mg./kg. orally 24 hr. after operation		Significance of treatment + = P < 0·01. ± = P, 0·01–0·05. − = P > 0·05				
	Emetine	Chiniofon, etc.	Emetine	Chiniofon	Stovarsol	Carbarsone	Diodoquin
Single dose therapy	20	1000	+	+	+	+	±
	10	500	+	+	−	−	−
	5	250	+	±	−	−	−
	2.5	125	+	±	..	..	..
	2.0	100	−	−	..	..	..
	orally 24, 30, 48, 54 and 72 hr. after operation						
Multiple dose therapy	5	1000	+	+	+	+	+
	2.5	500	+	+	±	+	+
	1.25	250	±	−	−	−	−
	0.62	125	±	−	..	−	..

DISCUSSION

In comparing the results of our experiments in rats with the results obtained in human amoebiasis with the same drugs, we must consider certain important differences between the infections in the two species. Whereas in rats the infection is an acute one and is reasonably standardized, in human amoebiasis there is not only the acute disease but also a chronic phase of widely varying symptomatology. In the latter form of the disease we may have also to deal with the cystic form of the parasite, against which there is as yet no suitable means of testing drugs experimentally. If we compare our results with the results usually obtained in acute amoebic dysentery in man we find reasonable agreement. Thus emetine and chiniofon appear to be the most effective, with stovarsol,

carbarsone, and diodoquin definitely inferior. If, however, we consider the respective merits of these drugs in chronic amoebiasis, we find them at variance with our experimental results, for against this form of the disease, carbarsone, stovarsol, and diodoquin are undoubtedly of value. The minimal effective therapeutic dose of this type of compound in rat and man differs considerably if the comparison is made on a mg./kg. basis. Thus a dose of 1,000 mg./kg., or 5 doses of 500 mg./kg., is required to produce an effect in rats, whereas the dosage used in humans, namely 4 gr. twice daily for 10 days (Manson-Bahr, 1945), corresponds to a total dosage of only 80 mg./kg. The validity of such a comparison, however, is questionable, and it is perhaps more reasonable to consider the relationship of the minimal effective therapeutic dose to the toxic dose in the two species. If this is done, the relationship is found to be approximately the same.

It is of interest to note the behaviour of diodoquin in our tests. When it was given as a single dose, its therapeutic effect was barely significant, and compared unfavourably with carbarsone and stovarsol. When it was given repeatedly, however, its activity was better demonstrated. The fact that it is poorly absorbed was no doubt responsible for this difference. This compound has been introduced comparatively recently (Hummel, 1939). It does not appear to be much more effective than the other amoebicides (Morton, 1945).

The strain of *E. histolytica* used in this series of experiments (isolated in culture from material kindly supplied by Dr. A. R. D. Adams) was one which produced infections susceptible to treatment with emetine. Not all strains do so, as has been mentioned in a previous paper (Jones, 1946). It was decided to use an emetine-susceptible strain for comparison as there was no evidence that the other drugs showed similar differences in effectiveness against different strains. The comparison was accordingly carried out under conditions equally favourable to all the test drugs.

Summary

A study has been made of the therapeutic action of emetine, chiniofon, stovarsol, carbarsone, and diodoquin against experimental amoebiasis in rats. Emetine and chiniofon appeared to have the widest range of activity. Stovarsol, carbarsone, and diodoquin were effective when given in large doses.

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